UNIVERSITY OF CALIFORNIA, SAN DIEGO

Recognition of Effectors by the Bacterial Type III Secretion System

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by

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The Dissertation of Loren E. Rodgers is approved,

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DEDICATION

This dissertation is dedicated to my loving wife Betsy.

Her patience is without end.

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ABSTRACT OF THE DISSERATION

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Pathogenic bacteria possess virulence proteins which are exquisitely tuned to modulate an array of targets. The type III secretion (TTS) system is broadly utilized by Gram-negative bacteria to deliver these effectors into host cells. *Yersinia pseudotuberculosis* delivers six virulence proteins, called "Yops," into host cells. Yops modulate the inflammatory response, cell survival, and phagocytosis – effective tactics which enable the bacterium to colonize phagocyte-rich lymph nodes. YopE is particularly well-studied, and disables members of the Rho protein family, which are critical to phagocytosis through their modulation of the actin cytoskeleton. Like many TTS effectors, translocation of YopE into host cells requires its prior

association with a dedicated chaperone protein within the bacterium. The mechanism by which this association promotes YopE translocation is the topic of this dissertation. I present evidence that SycE promotes a disorder-to-order transition within the chaperone-binding region of YopE. Among the secondary structure generated upon association between SycE and YopE is a β-hairpin in YopE, and I hypothesize that the ordering of specific residues within this domain serves to target the effector to a component of the TTS system required for translocation. Structures of diverse TTS effectors from several species reveal that this motif is conserved, indicating that this motif might function as a general targeting motif. I mutated a set of three or five residues within the YopE β-hairpin and find the translocation of the altered protein to be dramatically reduced. We demonstrate that these mutations do not destabilize the YopE-SycE interaction, and we propose that the ordering of these YopE residues by the chaperone SycE identifies the effector as a substrate for the TTS system. We seek to identify the receptor for this targeting signal, and we present preliminary results on purification of several proteins that might function as receptors for translocation targeting signals. Finally, recommendations are made for future investigations.

I.

Introduction

INTRODUCTION

A reciprocal struggle exists between infectious microbes and their hosts. The human immune system confronts bacterial pathogens with numerous barriers, and solutions to these defenses have been fine-tuned over evolutionary time, resulting in exquisite mechanisms capable of supporting diverse pathogenic lifestyles. Microbes frequently secrete virulence factors to modulate host targets, and delivery of these effectors may be accomplished by several means. The focus of this dissertation is the mechanism by which the type III secretion (TTS) system recognizes effectors as export substrates, and delivers these proteins into host cells.

A devastating example of TTS-mediated pathogenesis is presented by *Yersinia pestis*, the etiologic agent of plague. These Gram-negative bacteria are capable of thriving even in phagocyte-rich lymph nodes, producing inflamed buboes characteristic of the disease. This environment typically neutralizes foreign material through cellular uptake, yet *Yersinia* resist phagocytosis. *Yersinia's* evasion of the innate and adaptive defenses requires the TTS system, which utilizes a syringe-like apparatus to inject toxic effector proteins directly into the cytosol of host cells (Hueck 1998; Ghosh 2004; Viboud and Bliska 2005). These effectors, termed "Yops" for Yersinia outer proteins, gain access to the interior of host cells and modulate the inflammatory response, focal adhesion

proteins, cell survival and cytoskeleton dynamics (Aepfelbacher, Trasak et al. 2007). The effector YopE targets the Rho-GTPases RhoA, Rac-1 and Cdc-42 (Black and Bliska 2000; Von Pawel-Rammingen, Telepnev et al. 2000). These membrane-associated GTPases regulate the actin cytoskeleton and are inactivated by GTPase accelerating proteins (GAPs). YopE residues 100-219 comprise a GAP domain, and delivery of this effector into phagocytes inhibits actin polymerization, thereby disabling phagocytosis.

In addition to *Yersinia*, TTS systems are widely utilized by pathogens of animals and plants, including species of *Pseudomonas, Shigella, Salmonella, Chlamydia, Vibrio, Bordetella, Escherichia, Burkholderia, Erwinia, Ralstonia and Xanthomonas.* In each case, effectors must transverse not only the inner and outer membranes of the bacterium, but also those presented by targeted host cells. The central apparatus of the TTS system is the "injectisome," a structure composed of 20-25 protein. Genes for injectisome components are either encoded on virulence plasmids or clustered within pathogenicity islands on the bacterial chromosome (Hansen-Wester and Hensel 2001). Approximately half of the proteins comprising the injectisome are conserved between species. Most of the shared components are similar to those of the basal body of the flagella, which secretes subunits during assembly and is capable of secreting virulence factors (Young, Schmiel et al. 1999). Protein transport by both the pathogenic TTS system and the flagellar export system is dependent on the proton motive force (Galperin, Dibrov et al. 1982; Wilharm, Lehmann et al. 2004; Minamino and

Namba 2008; Paul, Erhardt et al. 2008). ATP hydrolysis at the inner membrane enhances export by both the TTS and flagellar systems, (Iino 1969; Emerson, Tokuyasu et al. 1970; Eichelberg, Ginocchio et al. 1994; Woestyn, Allaoui et al. 1994; Tamano, Aizawa et al. 2000) but is not absolutely required for export (Minamino and Namba 2008; Paul, Erhardt et al. 2008). The structural and functional similarities exhibited by the flagellar and pathogenic type III secretion systems has been explored through phylogenic analyses, and it was concluded that either the type III system evolved from the flagellar system (Nguyen, Paulsen et al. 2000; Saier 2004), or both systems evolved separately from a common ancestor (Gophna, Ron et al. 2003).

Most effectors bind specific chaperone proteins within the bacterium, and this association is required for translocation of effectors into host cells (Ghosh 2004). These chaperones are necessary for virulence, exemplified by a *Yersinia pseudotuberculosis* strain lacking the YopE chaperone SycE. This strain possesses a 450-fold lower LD_{50} than a wild type strain (Rosqvist, Forsberg et al. 1990). Each TTS chaperone is dedicated to the translocation of one or only a few effectors, and while such chaperones possess little primary sequence homology, they adopt remarkably similar structures (Fig. 1.1) (Birtalan and Ghosh 2001; Evdokimov, Tropea et al. 2001; Luo, Bertero et al. 2001; Stebbins and Galan 2001; Birtalan, Phillips et al. 2002; Phan, Tropea et al. 2004; Singer, Desveaux et al. 2004; van Eerde, Hamiaux et al. 2004; Buttner, Cornelis et al. 2005; Locher, Lehnert et al. 2005; Schubot, Jackson et al. 2005; Lilic, Vujanac et al. 2006).

Chaperones are compact (~14 kD), acidic (pI 4-5) proteins which form globular dimers, with four hydrophobic patches per dimer. Each protomer contributes one hydrophobic patch to the dimer interface and another patch to the chaperone-effector interaction (Birtalan and Ghosh 2001; Stebbins and Galan 2001; Birtalan, Phillips et al. 2002). Effectors form short α-helical and β-strand segments where they interact with these sites, and the majority of the ~50-residue chaperone-binding (Cb) region wraps around the chaperones in a dramatically extended fashion (Fig. 1.1) (Stebbins and Galan 2001; Birtalan, Phillips et al. 2002; Phan, Tropea et al. 2004; Schubot, Jackson et al. 2005; Lilic, Vujanac et al. 2006). The Cb region is flanked by two functional elements of the effector. Regions of the effectors that directly interact with targets within host cells, such as the YopE GAP domain, reside carboxy-terminal to the Cb domains. Amino-terminal to the Cb domain resides a translocation signal. This region is unlike the distinctive C-terminal glycine-rich repeats found in proteins exported by the type I secretion system (Delepelaire 2004) or the N-terminal peptides utilized by the type II (sec) system which are recognizable by a sequence or posititively charged, hydrophobic and then polar regions (Nielsen, Engelbrecht et al. 1997; Palmer, Sargent et al. 2005). The N-terminal type III secretion signal is proposed to consist of the first 15 amino acids or mRNA codons, or both (Lloyd, Sjostrom et al. 2002; Ghosh 2004; Ramamurthi and Schneewind 2005). N-terminal type III secretion signals are highly degenerate, demonstrated by the ability of alternating series of serines

and isoleucines to substitute for much of the YopE N-terminal secretion signal (Lloyd, Norman et al. 2001). The term "secretion" refers to export into culture media, whereas "translocation" denotes the transfer of effectors into the targeted host cell. The amino-terminal secretion signal of YopE is capable of driving the secretion of fused reporters (Sory, Boland et al. 1995; Schesser, Frithz-Lindsten et al. 1996; Anderson and Schneewind 1997; Anderson, Fouts et al. 1999; Ringdahl, Svensson et al. 2000; Lloyd, Norman et al. 2001), but translocation of Yops requires both the chaperone-binding domain and a cognate chaperone (Sory, Boland et al. 1995; Woestyn, Sory et al. 1996).

Chaperones are proposed to confer a competitive advantage by enabling their bound effectors to be translocated before effectors which do not partner with chaperones. Boyd *et al* demonstrated that while the chaperone-binding domain (Cb) of YopE is critical for translocation in a wild-type *Yersinia* strain, these residues are non-essential for translocation when other effectors are absent (ΔHOPEM) (Boyd, Lambermont et al. 2000). YopO is reported to have similar requirements for chaperone-mediated translocation (Letzelter, Sorg et al. 2006), suggesting that a major function of TTS chaperones is to facilitate the delivery of Yops to the injectisome. These observations form the basis of the translocation-targeting model of chaperone action, wherein the chaperone mediates the recognition of the effector by another TTS component. A structural comparison of the chaperone-binding domains of *Yersinia* YopE and *Salmonella* SptP reveal

that in spite of general sequence dissimilarity, chaperones induce the formation of similar stereochemical effector features that are hypothesized to identify the effector as a substrate for translocation (Stebbins and Galan 2001; Birtalan, Phillips et al. 2002).

An alternative hypothesis has been offered to explain the mechanism of action of TTS chaperones. Instead of identifying the effector as a substrate for translocation, chaperones are proposed to locally destabilize their bound effectors. One interpretation of the dramatically extended effector backbones seen in chaperone-effector complexes is that TTS chaperones maintain effectors in a partially unfolded conformation that primes the neighboring domains for unfolding and subsequent translocation (Stebbins and Galan 2001; Stebbins and Galan 2003). Concordantly, Feldman *et al.* found that the *Yersinia* TTS chaperone SycE was required for the secretion of a fusion of DHFR to a fragment of the YopE Cb domain, and a similar construct containing a less stable form of DHFR was secreted even in the absence of SycE (Feldman, Muller et al. 2002). Similar results have been obtained when native or destabilized ubiquitin was used as a YopE fusion partner (Lee and Schneewind 2002). In contrast, the ability of the TTS apparatus to secrete the amino-terminal 18 residues of YopE fused to GFP demonstrates that even highly stable C-terminal globular domains can be exported by the TTS apparatus without the need for chaperone-mediated unfolding (Jacobi, Roggenkamp et al. 1998). Globular effectors are unlikely to pass through the ~20 Å-diameter TTS needle (Blocker, Jouihri et al. 2001;

Hoiczyk and Blobel 2001) in a native state, and Akeda and Galan have demonstrated the ability of the peripherally-associated inner membrane *Salmonella* ATPase InvC to unfold a TTS effector *in vitro* (Akeda and Galan 2005). It remains unknown whether chaperones participate in the process of unfolding TTS effectors.

The primary aim of the research presented in this dissertation is to explain the mechanism by which TTS chaperones promote effector translocation. The *Yersinia pseudotuberculosis* SycE-YopE complex has been the subject of many investigations, and yet the mechanism by which chaperones promote effector translocation is not known. Presented here is a comparison of the effector YopE in its free and chaperone-associated state (chapter two). I identified effector residues that are ordered by the chaperone, and demonstrated that these are critical for YopE translocation into host mammalian cells (chapter three). I hypothesize that these residues serve as a targeting motif, and present preliminary investigations into this hypothesis (chapter four). These data built on the work of many investigators, and I present recommendations for further study.

FIGURES

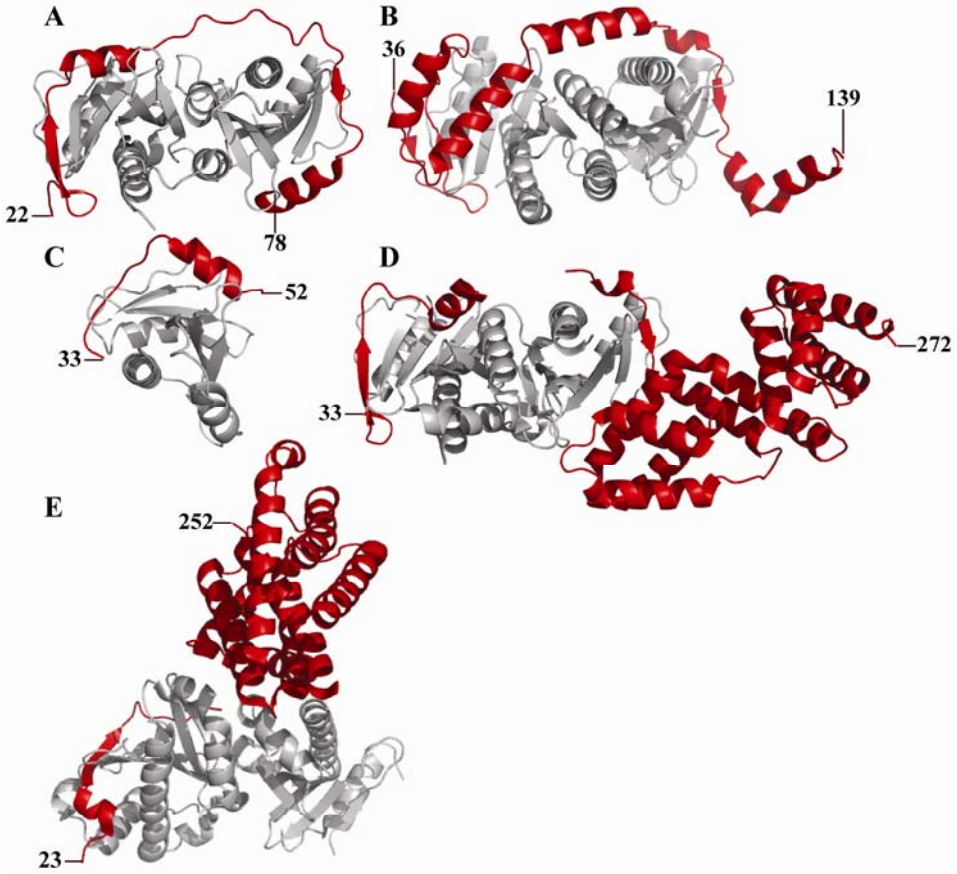

Figure 1.1. TTS effectors wrap around dimeric chaperones in an extended conformation.

Ribbon representation of TTS chaperone-effector complexes. Effectors are depicted in red, and chaperones are gray. The terminal amino acids of each effector fragment are indicated. Representations were generated using Pymol (DeLano 2002).

A. *Yersinia pseudotuberculosis* SycE (gray) binds YopE (red). Generated from PDB file 1L2W (Birtalan, Phillips et al. 2002).

B. *Salmonella typhimurium* SicP (gray) binds SptP (red). Generated from PDB file 1JYO (Stebbins and Galan 2001).

C. *Yersinia pestis* SycH (gray) binds YscM2 (red). Generated from PDB file 1TTW (Phan, Tropea et al. 2004).

D. *Yersinia pestis* SycN (gray) and YscB (gray) bind YopN (red). Generated from PDB file 1XKP (Schubot, Jackson et al. 2005).

E. *Salmonella typhimurium* InvB (gray) binds SipA (red). Generated from PDB file 2FM8 (Lilic, Vujanac et al. 2006).

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II.

The type III secretion chaperone SycE promotes a localized disorder-to-order transition in the natively unfolded effector YopE

ABSTRACT

Many virulence-related, bacterial effector proteins are translocated directly into the cytosol of host cells by the type III secretion (TTS) system. Translocation of most TTS effectors requires binding by specific chaperones in the bacterial cytosol, although how chaperones promote translocation is unclear. To address this issue, we studied the consequences of binding by the *Yersinia* chaperone SycE to the effector YopE by NMR. These studies examined the intact form of the effector, whereas prior studies have been limited to well-ordered fragments. We found that YopE had the characteristics of a natively unfolded protein, with its N-terminal 100 residues including its chaperone-binding (Cb) region flexible and disordered in the absence of SycE. SycE binding caused a pronounced disorder-to-order transition in the Cb region of YopE. The effect of SycE was strictly localized to the Cb region, with other portions of YopE being unperturbed. These results provide stringent limits on models of chaperone action, and are consistent with the chaperone promoting formation of a three-dimensional targeting signal in the Cb region of the effector. The target of this putative signal is unknown but appears to be a bacterial component other than the TTS ATPase YscN.

INTRODUCTION

A large number of Gram-negative bacterial pathogens translocate virulence-related effector proteins directly into the cytosol of eukaryotic host cells via the type III secretion (TTS) system (Cornelis 2006). Translocation of most effectors requires their prior association in the bacterial cytosol with specific dimeric chaperone proteins (Ghosh 2004). A large family of such chaperones exists, with individual chaperones functioning in the translocation of only a single or just a few corresponding effectors. Although chaperones have limited sequence identity (≤ 20%), their folds and modes of effector-binding are well conserved (Birtalan and Ghosh 2001; Luo, Bertero et al. 2001; Stebbins and Galan 2001; Birtalan, Phillips et al. 2002; Phan, Tropea et al. 2004; Singer, Desveaux et al. 2004; van Eerde, Hamiaux et al. 2004; Buttner, Cornelis et al. 2005; Locher, Lehnert et al. 2005; Schubot, Jackson et al. 2005; Lilic, Vujanac et al. 2006). Chaperone dimers provide rigid and globular surfaces, around which effectors wrap a ~25- to ~100-residue chaperone-binding (Cb) region in strikingly extended conformation (Stebbins and Galan 2001; Birtalan, Phillips et al. 2002; Phan, Tropea et al. 2004; Schubot, Jackson et al. 2005; Lilic, Vujanac et al. 2006). This conformation is generally similar among effectors despite lack of obvious sequence homology.

Two models have been proposed to explain the role of the extended conformation of the effector Cb region. In the first, the extended conformation is considered to maintain or prime unfolding of the effector (Stebbins and Galan

2001) for transport through the narrow ~20-30 Å diameter bore of the TTS needle (Blocker, Jouihri et al. 2001). In the second, the extended conformation is suggested to form a discrete three-dimensional signal targeting the chaperone-effector complex to a TTS component required for translocation (Birtalan, Phillips et al. 2002). Recent results demonstrating binding between a *Salmonella* chaperone-effector complex and the TTS ATPase as well as ATP hydrolysis-dependent unfolding of the effector by the ATPase are consistent with both unfolding and targeting models (Akeda and Galan 2005).

To provide further insight into mechanisms of chaperone action, we investigated the structural and dynamic changes an intact effector undergoes upon chaperone binding. Prior studies have been incomplete, being limited to crystallographic studies of structured portions of protein fragments. This has been due to the aggregation-prone nature of free intact effectors and the intractability of intact chaperone-effector complexes to crystallization. To circumvent these issues, we devised methods for obtaining free intact YopE, a well-studied *Yersinia pseudotuberculosis* effector, and intact SycE-YopE chaperone-effector complexes at concentrations and conditions suitable for nuclear magnetic resonance (NMR) spectroscopy.

In common with other effectors, YopE requires not only the Cb region (residues 23-78) for translocation (Sory, Boland et al. 1995), but also a second discrete region, termed here signal 1 (Fig. 2.1A, 'S1'). While one line of evidence indicates that S1 is composed of the N-terminal ~15 amino acids of

YopE (Lloyd, Norman et al. 2001), other results indicate it is composed of the first ~15 mRNA codons of YopE (Ramamurthi and Schneewind 2005). These possibilities are not exclusive, and indeed ribonucleic and proteinaceous signals many be important at different times after host cell contact (Ghosh 2004). Following the S1 and Cb regions in effectors are host cell interaction domains, which in YopE consists of a single functionality. Residues 100-219 of YopE have RhoGAP activity (Fig. 2.1A) (Von Pawel-Rammingen, Telepnev et al. 2000), which is required for disrupting host cell actin and antagonizing phagocytic uptake of *Y. pseudotuberculosis* by macrophages.

The studies reported here revealed that the N-terminal 100 residues of YopE, including the S1 and the Cb regions, were disordered and flexible in the absence of bound SycE, as is characteristic of natively unfolded proteins. SycE binding brought about a pronounced disorder-to-order transition in the Cb region but had no effect on other portions of YopE. These results provide support for a targeting model of chaperone action, and are inconsistent with unfolding models. No association between SycE-YopE and the *Yersinia* TTS ATPase YscN was detected, suggesting that the target of the putative signal in the SycE-YopE complex is a *Yersinia* component other than YscN.

MATERIALS AND METHODS

Protein expression and purification

His-tagged SycE-YopE complexes were prepared as previously described (Birtalan, Phillips et al. 2002), except as follows. For production of ^{15}N-, ^{13}C-, or dually-labeled SycE-YopE, bacteria were grown at 37 ºC to mid-log phase in minimal medium containing ^{15}N $(NH_4)_2SO_4$ (1 g/L), ^{13}C glucose (2 g/L), or both, induced with 1 mM IPTG, and grown for 18 hours at 25 ºC. For production of triple labeled (^{2}H, ^{15}N ^{13}C) SycE-YopE, bacteria were grown in 2X-YT at 37 ºC to mid-log phase, at which point bacteria were harvested by centrifugation (10,000 x g, 20 min, 4 ºC), washed twice in M9 salts, and resuspended in one-fourth volume of minimal medium containing D_2O, ^{15}N $(NH_4)_2SO_4$, and ^{13}C glucose. Bacteria were then grown for 20 hours at 25 ºC.

Bacteria were harvested by centrifugation and lysed by sonication in lysis buffer (500 mM NaCl, 50 mM sodium phosphate, pH 8.0, 5 mM imidazole, 5 mM $MgCl_2$, and 10 mM β-mercaptoethanol supplemented with 1 mM PMSF and 20 µg/mL DNAse). The lysate was clarified by centrifugation (20 min, 30,000 x g, 4 ºC), and SycE-YopE was applied to a Ni^{2+}-chelation column and eluted using a 5-500 mM imidazole gradient in lysis buffer. The eluate was concentrated by ultrafiltration (MWCO 30 kDa), and further purified by size-exclusion chromatography (Superdex 75) in 500 mM NaCl, 50 mM sodium phosphate, pH 8.0, 1 mM DTT.

The YopE(RhoGAP) fragment (residues 81-219), containing a C-terminal his-tag (LEHHHHHH), was expressed using pET28b (Novagen), and produced with isotopic labeling and purified as above.

A biotinylated version of SycE-YopE, with YopE containing a C-terminal 21-residue biotinylation sequence (KLPAGGGLNDIFEAQKIEWHE), was expressed using pAC-6 in *E. coli* AVB101 cells (Avidity). *In vivo* biotinylated SycE-YopE was purified as previously described (Birtalan, Phillips et al. 2002), except that precipitation was carried out with 50% $(NH_4)_2SO_4$ and the protein was dialyzed in pH 8 Tris buffer.

Denaturation and renaturation

SycE-YopE was denatured in 8 M urea, 50 mM sodium phosphate, pH 8.0, and bound to Ni^{2+}-NTA agarose beads. Denatured YopE was eluted from these beads with 8 M urea, 50 mM sodium phosphate, pH 4.0, and renatured by dilution in 1 M arginine, 500 mM NaCl, 50 mM Tris, pH 8.0, 1 mM DTT, 5 mM EDTA at a final concentration of 1 μM (determined by A_{280}). For renaturation of SycE-YopE complexes, two-fold molar excess of denatured, unlabeled SycE was included with YopE. SycE was isolated from the unbound fraction of unlabeled, denatured SycE-YopE applied to Ni^{2+}-NTA agarose beads, or purified in native form as described previously (Birtalan and Ghosh 2001). Renaturation solutions containing YopE alone or YopE mixed with SycE were stirred vigorously for a minimum of 1 hr at 4 ºC, and then dialyzed in 500 mM NaCl, 50 mM Tris pH 8.0,

1 mM DTT, and 5 mM EDTA. Renatured and aggregated proteins were separated by size-exclusion chromatography (Superdex 200) in 50 mM sodium phosphate, pH 6.1.

NMR data collection

NMR spectra for SycE-YopE, free YopE, and YopE(RhoGAP) were typically measured at protein concentrations of 15-60, 2-4, and 45 mg/mL, respectively. Samples for NMR analysis were prepared in 45 mM sodium phosphate buffer, pH 6.1 (95% H_2O, 5% D_2O), and all NMR spectra were collected at 298 K and 700 MHz ^1H frequency on a Bruker Avance spectrometer with a cryoprobe. Sequential assignment was analyzed with the program CARA (Keller 2004), and stretches of sequentially connected spin systems were mapped to the amino acid sequence of YopE with the program MAPPER (Guntert, Salzmann et al. 2000). For free YopE all but four of 191 resonances were assigned, and for SycE-YopE all but 25 resonances were unassigned, of which about half are from the RhoGAP domain. $\Delta\,\delta Av$ represents $\sqrt{\left[\left(\Delta^1 H\right)^2 + 0.2\left(\Delta^{15} N\right)^2\right]/2}$.

Binding experiments with biotinylated SycE-YopE and YopE

Biotinylated SycE-YopE (650 μg) was incubated with 100 μL streptavidin-agarose beads for 1 hour at 25 ºC in 500 μL binding buffer (150 mM NaCl, 50mM sodium phosphate buffer, pH 8.0, 5% glycerol, 1% Triton X-100, 10mM β-mercaptoethanol), and beads were washed three times with 700 μL of

binding buffer. A portion of the streptavidin-agarose beads containing SycE-YopE was washed 15 times with 700 µL of 10 mM glycine, pH 2.8 to remove SycE. YopE remaining on beads was renatured by washing 5 times with binding buffer. This procedure completely removed SycE and resulted in only slight loss of YopE, as assayed by Western blotting and silver staining.

Lysates were prepared from *Y. pseudotuberculosis* 126 and *Y. pseudotuberculosis* 126 (*ΔyopE*). This latter strain was generated through standard allelic exchange procedures, with the coding region for *yopE* being replaced by the coding region for *kanR* (chapter three). Wild-type and *ΔyopE Y. pseudotuberculosis* were grown in BHI media at 37ºC until mid-log density, and type III secretion was induced by addition of sodium oxalate and $MgCl_2$ to final concentrations of 20 mM. After 3 hr, bacteria were harvested by centrifugation (10,000 x g, 20 min, 4 ºC) and resuspended in 25 mL binding buffer (supplemented with 1 mM PMSF and 0.5 mM E-64) per liter of bacterial culture, and lysed by sonication. The lysate was clarified by centrifugation (20 min, 30,000 x g, 4 ºC) and endogenous biotinylated proteins were removed by incubation with 400 µL streptavidin-agarose beads (Pierce) for 1 hour at 4 ºC, followed by centrifugation (5 min, 3500 x g, 4 ºC).

Lysates (~333 µL) from wild-type *Y. pseudotuberculosis* cultures (~8 mL) were added to 100 µL streptavidin-agarose beads containing bound SycE-YopE; lysates from the *ΔyopE* strain were added to streptavidin-agarose beads containing only bound YopE. Resulting slurries were rocked for 95 min at 4 °C, washed

three times for wild-type lysate and 30 times for *ΔyopE* lysate with 700 μL binding buffer. Bound proteins were removed from beads by boiling in 2x SDS-PAGE sample buffer, resolved by 12% SDS-PAGE, and transferred to a PVDF membrane. Membranes were probed with anti-YscN (gift of O. Schneewind) (Blaylock, Riordan et al. 2006) or anti-SycE rabbit polyclonal antibodies, whose binding was visualized using HRP-conjugated goat anti-rabbit secondary antibodies (Santa Cruz) and ECL plus (Amersham). Anti-SycE antibodies were produced in rabbits by standard means using purified SycE as an antigen (Antibodies Incorporated).

RESULTS

Renaturation of free and SycE-bound YopE for NMR studies

To carry out NMR characterization of the 52 kDa SycE-YopE complex, selectively labeled SycE-YopE complexes were prepared by a denaturation-renaturation protocol. Such complexes contained unlabeled SycE and isotopically labeled (with ^{15}N, ^{13}C, ^{2}H, or a combination of these) YopE. For this procedure, biosynthetically labeled SycE-YopE complexes were recombinantly expressed and purified. Labeled YopE (23 kDa) was dissociated from labeled dimeric SycE (29 kDa per dimer) by urea-induced denaturation, and then renatured with unlabeled SycE in a non-chaotropic buffer to produce selectively labeled SycE-YopE. Free YopE was generated similarly, except that SycE was omitted during renaturation.

Renatured SycE-YopE was verified to have circular dichroism spectra and gel filtration chromatography profiles indistinguishable from those of catalytically active complexes isolated in native form (i.e., without denaturation, data not shown) (Birtalan, Phillips et al. 2002). However, NMR analysis provided the most precise verification for renatured SycE-YopE and free YopE. ^{15}N-^{1}H transverse relaxation optimized spectroscopy (TROSY) spectra of renatured YopE and SycE-YopE (Fig. S1) were acquired and compared to that of YopE(RhoGAP), a truncated form of YopE. This truncated form contains only the RhoGAP domain (residues 81-219), and was isolated in native form (i.e.,

without denaturation steps). A similar fragment (residues 90-219) was crystallized and demonstrated to have RhoGAP catalytic activity (Von Pawel-Rammingen, Telepnev et al. 2000; Evdokimov, Tropea et al. 2002). Without exception, all 147 cross peaks in the ^{15}N-^{1}H TROSY spectrum of YopE(RhoGAP) superimposed with cross peaks in the spectrum of renatured YopE as well as that of SycE-YopE. This complete superimposition indicated that renatured YopE and SycE-YopE had properly folded conformations in their RhoGAP domains and were suitable for structural analysis.

No effect of SycE binding on the YopE RhoGAP domain

^{15}N-^{1}H TROSY spectra of YopE and SycE-YopE were compared to evaluate the effects of SycE binding on YopE. We observed 191 cross peaks for the ^{15}N-^{1}H-moieties of free YopE and 244 for SycE-bound YopE (Fig. 2.1B). The 244 YopE resonances in SycE-YopE were in excess of the 214 resonances expected (including a His-tag, see Materials and Methods), suggesting the existence of multiple conformations and possibly the incomplete suppression of side chain resonances. More importantly, the great majority of free YopE resonances (151 of 191) overlapped (< 0.1 ppm change) with SycE-YopE resonances (Fig. 2.1B), which indicated that SycE binding had a limited effect on YopE. The 40 YopE resonances that had no counterparts in the SycE-YopE spectrum (i.e., those shifted upon SycE binding) were also notably absent in the spectrum of the YopE(RhoGAP) fragment. This indicated that SycE exerted its

effect within the N-terminal 80 residues of YopE and had no effect on the RhoGAP domain.

Localized effect of SycE on YopE

To characterize the effects of SycE on YopE at the resolution of individual residues, we assigned cross peaks in ^{15}N-^{1}H-TROSY spectra to corresponding residues. We acquired sequential backbone assignments for ^{2}H,^{13}C,^{15}N-labeled YopE in its free or SycE-bound states using 3D TROSY-HNCA, 3D TROSY-HNCACB, and a 3D ^{15}N-resolved [^{1}H,^{1}H]-NOESY (with 100 ms mixing time) for the collection of ^{1}HN-^{1}HN NOE's (Bax and Grzesiek 1993; Salzmann, Pervushin et al. 1999; Salzmann, Wider et al. 1999; Xia, Sze et al. 2000).

For free YopE, 91% of possible assignments were made. Residues lacking assignments were: Met1 and Lys2 in S1; Leu55-Arg58, Arg62-Ile71, Phe73, Ile74, and Phe78 in Cb; and Gln149 in the RhoGAP domains. These gaps in the Cb region were likely due to overlapping resonances. For SycE-bound YopE, 95% of possible assignments were made. Residues lacking assignments were: Met1 and Lys2 in S1; Ser36, Asn41, Leu43, Ser64, Ser69, Phe73, and Arg76 in the Cb region; and Gln149 in the RhoGAP domain. The assignment process for SycE-YopE was complicated by the fact that ~25% of residues in the Cb region showed duplicated chemical shifts, and several residues in this region showed very weak peak intensities, in particular the segment comprising residues 67-78. Duplicated resonances likely arose from local conformational exchange

induced by proline cis-trans isomerization, conformational heterogeneity in regions with poorly defined secondary structure, or both. These characteristics are consistent with high temperature-factors for the Cb region in the crystal structure of SycE-YopE(Cb) (Birtalan, Phillips et al. 2002), especially in the α-helical region formed by residues 67-78.

Thirty-five of the 40 [^{15}N,^{1}H]-TROSY cross peaks of free YopE that were shifted upon SycE binding were assigned in both free and chaperone-bound states. Notably, these shifted and dually assigned cross peaks all mapped to residues in the Cb region (Fig. 2.1C). The magnitude of these shifts was large, indicating that a major structural transition had been brought about by SycE binding. Four residues within the Cb region had resonances that were unshifted: Gln51, Ser65, Val66, and Ala67. The crystal structure of the SycE-YopE(Cb) fragment shows that these residues are not contacted by SycE and lack regular secondary structure (Birtalan, Phillips et al. 2002). Comparison of dually assigned residues also revealed that SycE binding had no effect on the S1 region and likewise no effect on residues 81-100 connecting the Cb region to the RhoGAP domain. These results provide direct evidence for the effect of SycE binding being strictly localized to the Cb region of YopE, and not extending outside the Cb region.

Random coil in the Cb region of free YopE

We next addressed the issue of whether the Cb region of free YopE was in a folded state. Backbone chemical shifts of free YopE were compared with those

expected for a random coil state. The deviation from random coil values of secondary chemical shifts (Δδ) in ^{15}N, ^{1}H, and ^{13}C dimensions of the Cb region of free YopE were found to be close to zero, indicating that this region was in a random coil state (Fig. 2.2). In contrast when SycE was bound, these same residues of YopE had values significantly different from those typical for a random coil state (Fig. 2.2, Δδ). The deviation from random coil values of $^{13}C\alpha$ chemical shifts in SycE-bound YopE suggested α-helical secondary structure for Asp37-Leu43 and Ala67-Ser79 and β-strand secondary structure for Gly24-Gln34, Glu48-Gly52, and Arg58-Leu63, agreeing almost exactly with the crystal structure of the SycE-YopE(Cb) complex (Birtalan, Phillips et al. 2002). The only exception was Glu48-Gly52, which lacks β-strand characteristics in the crystal structure. These results provide evidence for SycE promoting a transition in the Cb region from an unstructured state to a structured conformation.

As expected, there were large deviations from random coil values in secondary chemical shifts of the RhoGAP domain for both free and SycE-bound YopE (Fig. 2.2). $^{13}C\alpha$ chemical shifts for this region were in close agreement with the crystal structure of this domain (Evdokimov, Tropea et al. 2002). Residues 79-101 connecting the Cb region to the RhoGAP domain lacked detectable secondary structure regardless of SycE binding (Fig. 2.2), indicating that these residues likely serve as an unstructured linker.

Disorder-to-order transition upon SycE association

We next examined dynamic properties of free YopE to ask whether the Cb region, despite having random coil-like chemical shifts, was discretely structured. A heteronuclear $^{15}N\{^{1}H\}$-NOE experiment was carried out to examine the mobility of amide $^{1}H^{N}$-^{15}N bond vectors (Kay, Torchia et al. 1989). In this experiment values close to 1 correspond to $^{15}N,^{1}H$-moities that are well structured (i.e., values of 0.8-1.0 are indicative of a rotational correlation time that is greater than a few ns), and values < 0.5 are indicative of an unstructured and flexible state (i.e., values < 0 are indicative of a rotational correlation time < 1 ns). The $^{15}N,^{1}H$-moieties of the RhoGAP domain had $^{15}N\{^{1}H\}$-NOE values close to 1 in both free and SycE-bound YopE (Fig. 2.3A), indicating that the RhoGAP domain was well structured, both in the presence and absence of SycE. In contrast, the N-terminal 98 residues of free YopE had markedly lower $^{15}N\{^{1}H\}$-NOE values (Fig. 2.3A), diagnostic of an unstructured and flexible state. The $^{15}N\{^{1}H\}$-NOEs of the Cb region were between 0.0-0.4, which indicated that this region had some local and limited ordering.

Upon addition of SycE to free YopE, $^{15}N\{^{1}H\}$-NOE values in the Cb region (i.e., residues 22-83) rose considerably (Fig. 2.3A), providing evidence that the Cb region had become structurally constrained by SycE. Similarly, the intensities of the cross peaks in the $[^{15}N,^{1}H]$-TROSY spectrum throughout the Cb region were significantly elevated in free YopE as compared to SycE-bound YopE, consistent with a disordered and flexible state in this region when free but structurally constrained when SycE-bound (Fig. 2.3B). These results

demonstrated that the Cb region was unstructured and flexible in free YopE but nevertheless competent to bind SycE, and that SycE binding caused a pronounced disorder-to-order transition within this region.

Disorder of S1 with or without SycE

The cross peaks of the [^{15}N,^{1}H]-TROSY spectra for the N-terminal 20 residues in free and SycE-bound forms of YopE superimposed well, indicating that SycE had no effect on the S1 region (Fig. 2.1). Based on the random coil-like chemical shifts (Fig. 2.2) and the negative ^{15}N{^{1}H}-NOE values (Fig. 2.3), the S1 region appeared to be unstructured and flexible in both free and in SycE-bound YopE. It is noteworthy that in the flagellar export system, which is related to TTS systems, the N-terminal secretion signal of exported flagellar proteins are also disordered (Daughdrill, Chadsey et al. 1997).

No association between SycE-YopE and YscN

A disorder-to-order transition imposed by SycE supports a targeting mechanism of chaperone action. Since association of chaperones, effectors, and chaperone-effector complexes with TTS ATPases have been detected in *Salmonella* and *E. coli* (Gauthier and Finlay 2003; Akeda and Galan 2005), we sought to determine whether SycE-YopE, free YopE, or both associated with the *Y. pseudotuberculosis* TTS ATPase YscN (48 kDa). A detergent-solubilized *Y. pseudotuberculosis* lysate was prepared using a protocol similar to that used for

detecting association in *Salmonella* and *E. coli*. The lysate was applied to streptavidin-agarose beads containing SycE-YopE complexes immobilized via site-specific biotinylation of YopE. While YscN was evident in the solubilized lysate and in material that did not adhere to the beads, no YscN was detected bound to SycE-YopE (Fig. 2.4A). As a positive control, a detergent-solubilized lysate from *Y. pseudotuberculosis* (*ΔyopE*) was applied to biotinylated free YopE immobilized on streptavidin-agarose beads, and SycE was found to specifically associate with free YopE as expected (Fig. 2.4B). Again, no YscN was detected (data not shown), even though ~13-fold more sample was applied to the YscN immunoblot as compared to the SycE immunoblot. We also did not detect interaction of SycE-YopE with purified oligomeric YscN that had been expressed in *E. coli* and verified to have ATPase activity (K. Bzymek and P.G., unpublished results).

DISCUSSION

TTS effectors have been observed to associate with their specific chaperones through a conserved binding mode. The most distinctive feature of this mode is the highly extended conformation of the effector Cb region, as seen for fragments of *Salmonella* SicP (Stebbins and Galan 2001) and SipA (Lilic, Vujanac et al. 2006) and *Yersinia* YopE (Birtalan, Phillips et al. 2002), YscM2 (Phan, Tropea et al. 2004), and YopN (Schubot, Jackson et al. 2005) bound to their specific chaperones. However, the functional importance of this binding mode has remained unclear. To inform models of chaperone action, we investigated the consequences of SycE binding on the dynamics and structure of intact YopE through NMR studies.

Three major conclusions derived from these studies. First, the effect of SycE binding was strictly localized and isolated to the Cb region of YopE, as deduced from comparison of [^{15}N,^{1}H]-TROSY spectra (Fig. 2.1). Binding by SycE had no effect on the YopE S1 region, the RhoGAP domain, or the linker region connecting Cb to the RhoGAP domain. Second, the Cb region in absence of SycE appeared to be flexible and disordered, as evidenced by chemical shift analysis, ^{15}N{^{1}H}-NOEs, and [^{15}N,^{1}H]-TROSY signal intensities (Fig. 2.3). It must be noted that the Cb region of free YopE had ^{15}N{^{1}H}-NOEs values between 0-0.4 (Fig. 2.3), which suggests some amount of local and limited ordering in this region. Such features may contribute to reducing the entropic cost

of binding SycE. These data also revealed that nearly half of free YopE was unstructured and flexible (Fig 5), thus identifying YopE as a member of the natively unfolded protein family (Fink 2005). In host cells, the disordered state of the N-terminal region of YopE may be required for association with host cell membranes (Krall, Zhang et al. 2003), as has been suggested for several effectors (Letzelter, Sorg et al. 2006). Third, as also revealed by chemical shifts, $^{15}N\{^{1}H\}$-NOEs, and [^{15}N,^{1}H]-TROSY NMR signal intensities, a pronounced disorder-to-order transition in the YopE Cb region was brought about by SycE binding.

These conclusions are at odds with models of chaperone action that require consequences outside the Cb region, for example in priming unfolding of C-terminal host-interaction domains or ensuring proper presentation of the S1 region. These conclusions are also inconsistent with models in which chaperone binding maintains the Cb region in an unfolded state (Stebbins and Galan 2001). Rather than maintaining an unfolded state in the effector, SycE was found to promote structuring of the YopE Cb region. Taken together with structural conservation of chaperone-effector complexes, our results are most consistent with a targeting model of action in which the Cb region in association with the chaperone constitutes a three-dimensional targeting signal (Birtalan, Phillips et al. 2002). The strongest experimental support for the targeting model comes from a *Yersinia* strain deleted of most of its effectors (*ΔHOPEM*) (Boyd, Grosdent et al. 2000). In this strain, neither SycE nor the Cb region of YopE is required for

translocation, whereas in wild type strains both are required. Dispensability of SycE and the Cb region in the *ΔHOPEM* strain suggests that chaperones have a role in enhancing interaction between effectors and a TTS component that is required for translocation and that is also limiting in a wild-type background (i.e., in the presence of other chaperone-effector complexes). Additional evidence for competition between chaperone-effector complexes exists (Wulff-Strobel, Williams et al. 2002).

Whether this potentially limiting component is the TTS ATPase is not clear. In *Salmonella*, the SicP-SptP chaperone-effector complex interacts with the TTS ATPase InvC, but the chaperone SicP is sufficient for this interaction (Akeda and Galan 2005). Likewise, in *E. coli* the chaperone CesT is sufficient for interaction with the TTS ATPase EscN, and so too is the effector Tir (Gauthier and Finlay 2003). Based on these data, it seems improbable that the TTS ATPase is the receptor for a putative translocation signal carried by the chaperone-effector complex, unless such complexes bind more strongly to the TTS ATPase than isolated chaperones and effectors. The relative affinities of chaperone-effector complexes as compared to individual components are not known. In *Yersinia,* interaction between SycE-YopE and the TTS ATPase YscN was not detected, suggesting that the putative receptor for a targeting signal in the SycE-YopE complex is a bacterial component other than YscN.

FIGURES

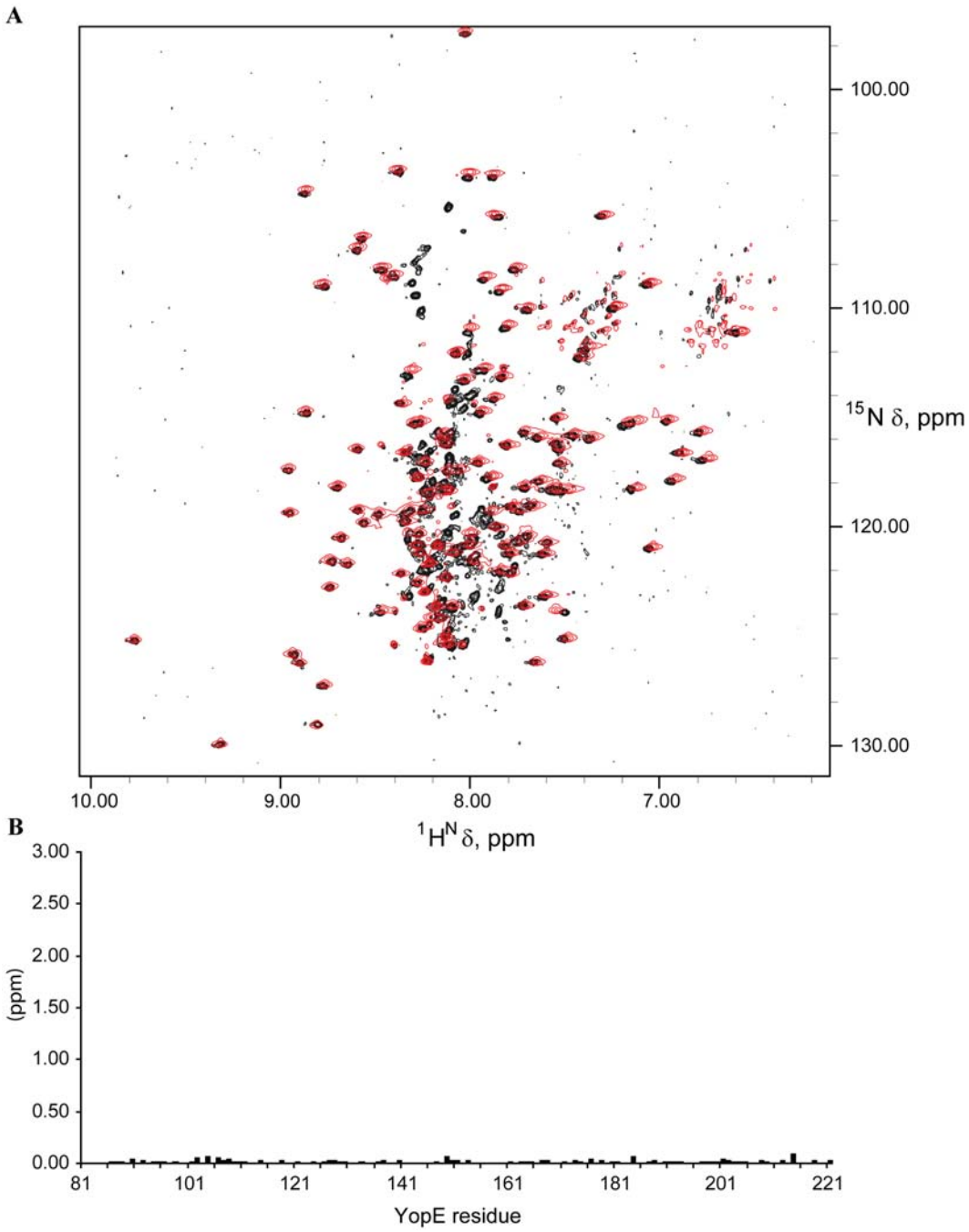

Figure 2.1. Structural integrity of renatured YopE and SycE-YopE.
A. [15N,1H]-TROSY NMR spectra of renatured 15N-labeled YopE (black) and 15N-labeled YopE(RhoGAP) (residues 81-219) (red).
B. Differences in 1H and 15N chemical shifts (δ ΔAv) between YopE and YopE(RhoGAP).

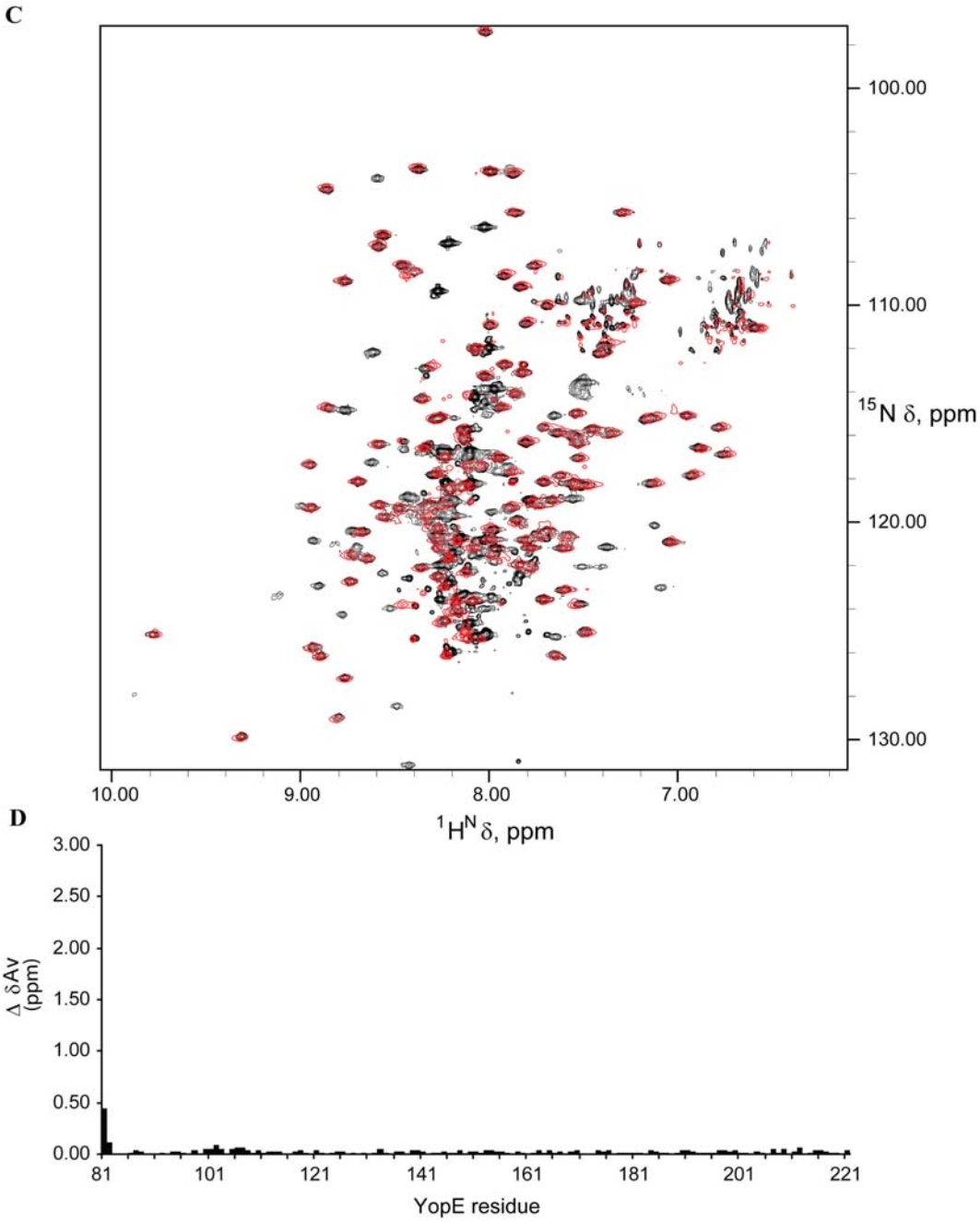

Figure 2.1 continued.

C. [15N,1H]-TROSY NMR spectra of renatured SycE-15N-lableled YopE (black) and 15N-labeled YopE(RhoGAP) (red).

D. Differences in 1H and 15N chemical shifts (δ ΔAv) between SycE-YopE and YopE(RhoGAP).

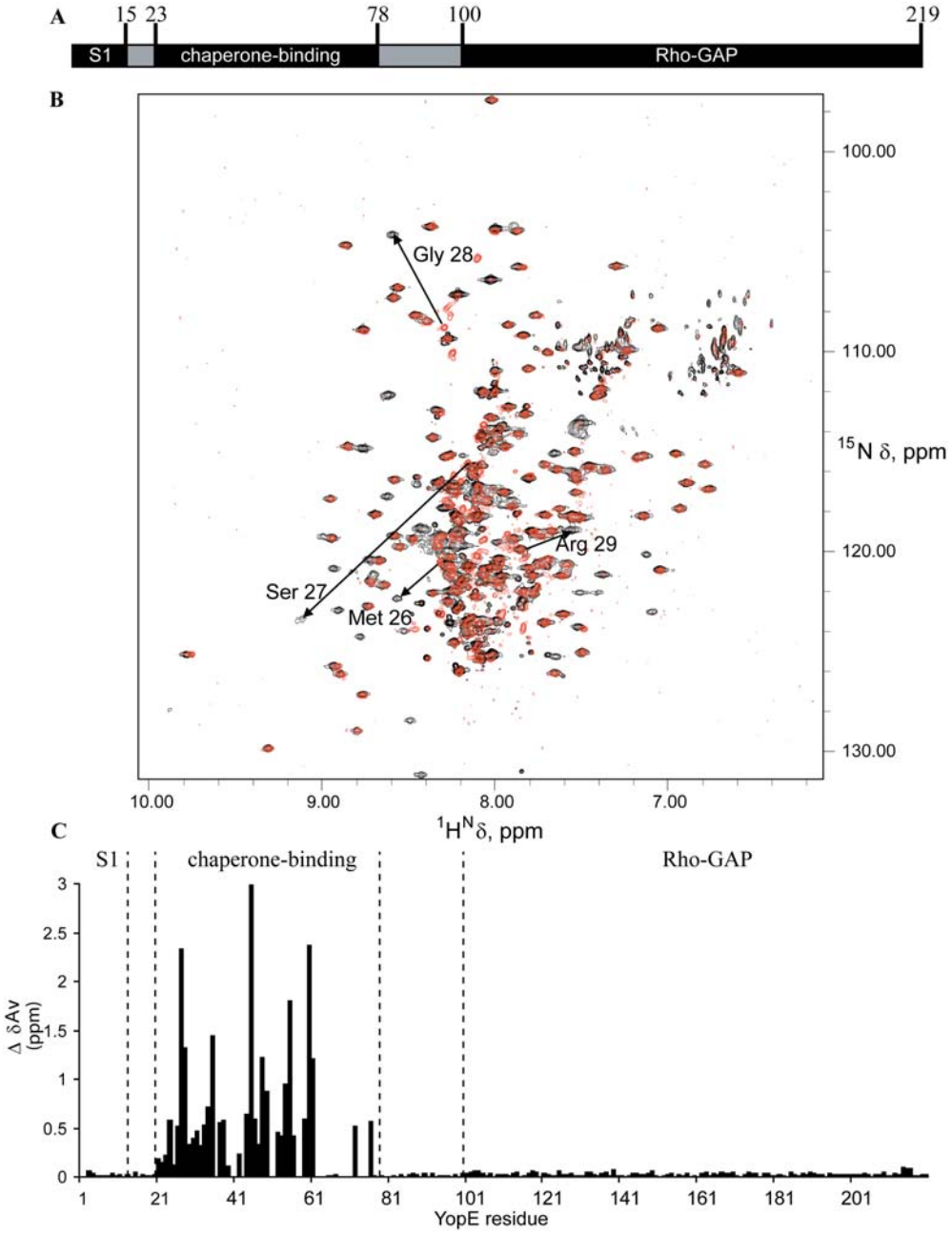

Figure 2.2. Effect of SycE binding confined to the Cb region of YopE.
A. Functional YopE regions: signal 1 (S1), chaperone-binding (Cb) region, and RhoGAP domain.
B. [^{15}N,^{1}H]-TROSY NMR spectra of free (red) and SycE-bound (black) ^{15}N,^{2}H-labeled YopE. Arrows show examples of YopE cross peaks differing between free (tail of arrow) and SycE-bound forms (arrowhead).
C. Difference between chemical shifts (ΔδAv) for the spectrum in panel B assigned to YopE residues.

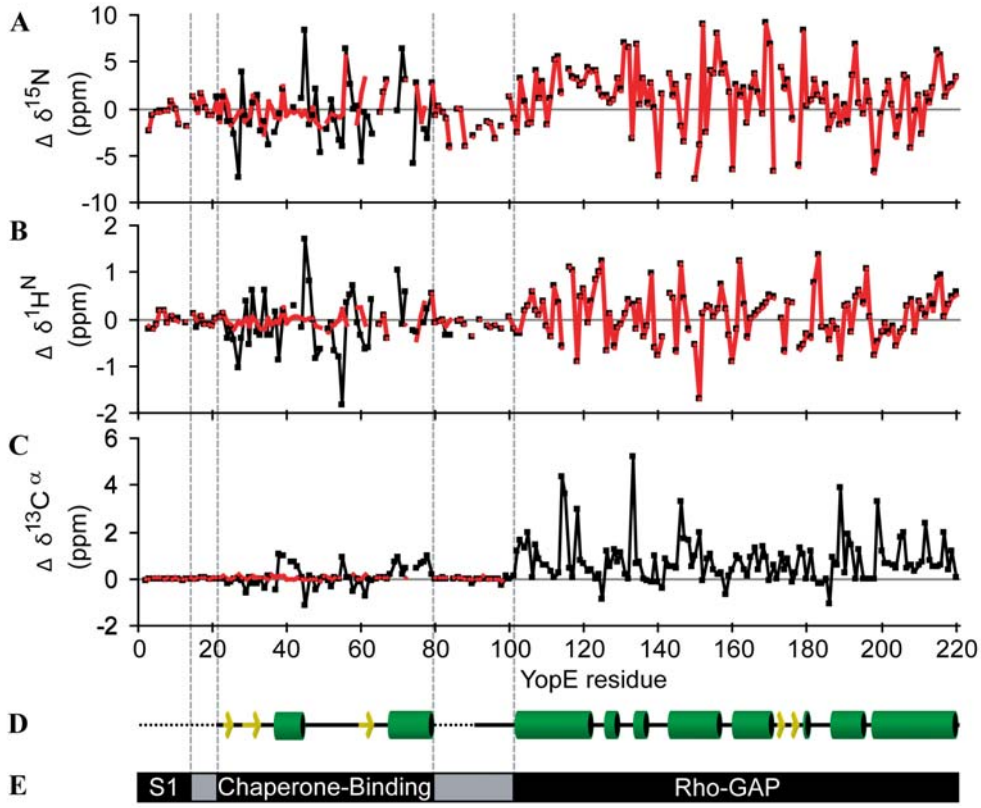

Figure 2.3. Random coil conformation of the N-terminal half of free YopE. Chemical shift deviations from those expected for random coil conformation (Δδ secondary chemical shifts) of **A** ^{15}N **B**, ^{1}H, and **C** ^{13}Cα nuclei for free (red) or SycE-bound (black) ^{15}N,^{2}H,^{13}C-labeled YopE, after application of sequence-dependent corrections (Schwarzinger, Kroon et al. 2001). ^{13}Cα secondary chemical shifts were not observed for residues of the RhoGAP domain of free YopE, due to low sensitivity of the experiment, low sample concentration, and the highly structured nature of these residues.
D. Secondary structure of YopE from crystal structures of SycE-YopE(Cb) (Birtalan, Phillips et al. 2002) and RhoGAP (Evdokimov, Tropea et al. 2002) domain aligned with secondary chemical shifts. Yellow arrows depict β-strands; green cylinders, α-helices; and black lines, loops. Regions not present in crystal structures are depicted as dotted lines.
E. Functional regions of YopE aligned with secondary chemical shifts and secondary structure.

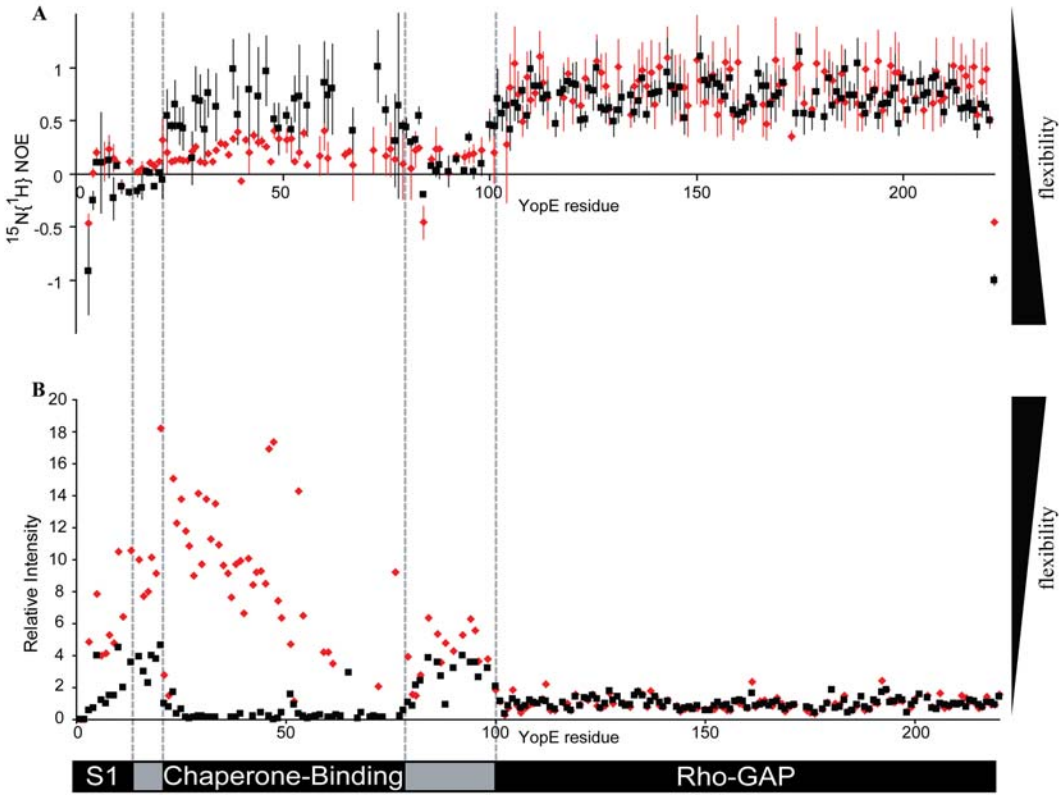

Figure 2.4. Dynamics of free and SycE-bound YopE.
A. ^{15}N{^{1}H}-NOE intensity values are plotted for free (red) and SycE-associated (black) ^{15}N,^{2}H-labeled YopE. Error bars were calculated from the spectral noise of the reference experiments.
B. [^{15}N,^{1}H]-TROSY cross peak intensities are plotted for free (red) and SycE-bound (black) ^{15}N-labeled YopE, with intensities normalized to the mean intensity of resonances from the RhoGAP domain. Samples were prepared by adding either phosphate buffer or unlabeled SycE to otherwise identical aliquots of free ^{15}N-labeled YopE.

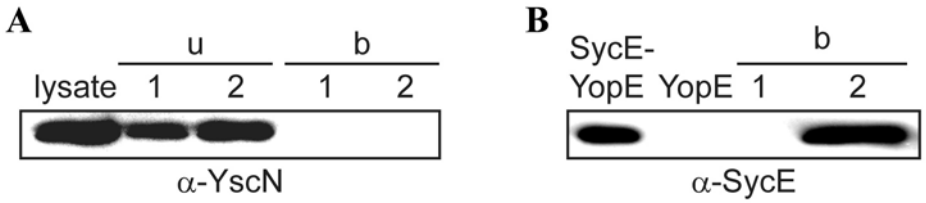

Figure 2.5. Lack of association between SycE-YopE and YscN.
A. YscN was present in a *Y. pseudotuberculosis* detergent-solubilized lysate ('lysate'), as detected by immunoblot with αnti-YscN polyclonal antibodies. The lysate was applied to unmodified streptavidin-agarose beads ('1') or streptavidin-agarose beads with biotinylated SycE-YopE attached ('2'). YscN was present in unbound fractions but not in bound fractions for beads '1' and '2'.
B. SycE, as detected by immunoblot with anti-SycE polyclonal antibodies, from a detergent-solubilized lysate of *Y. pseudotuberculosis* (*ΔyopE*) did not bind unmodified streptavidin-agarose beads ('1'), but did bind streptavidin-agarose beads with biotinylated free YopE attached ('2'). As controls, SycE was detected in biotinylated SycE-YopE complexes ('SycE-YopE') but not in biotinylated free YopE ('YopE').

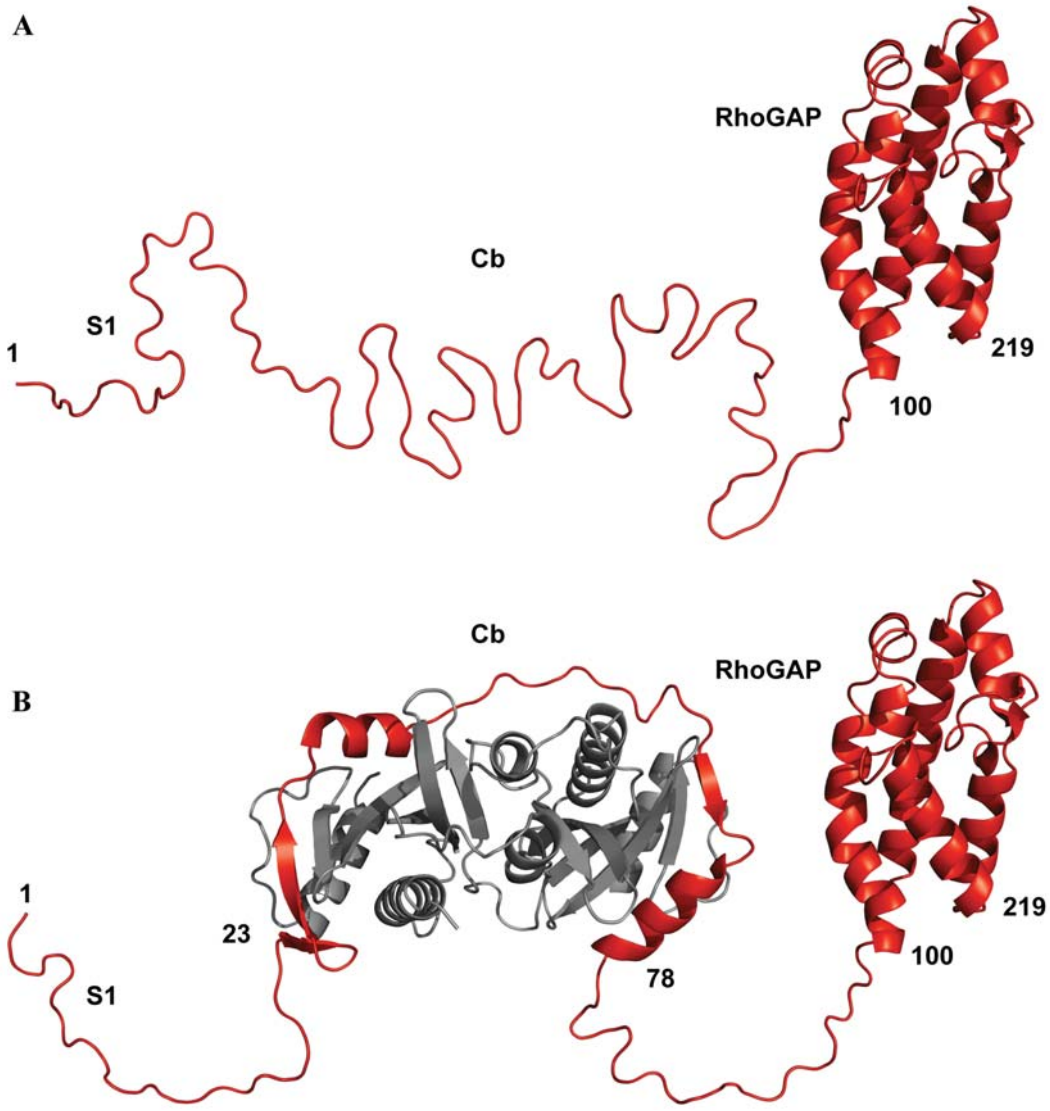

Figure 2.6. Free and SycE-bound YopE.
A. Ribbon representation of free YopE, with the crystal structure of the RhoGAP domain (Evdokimov, Tropea et al. 2002) depicted and other portions in random coil conformation.
B. Ribbon representation of the SycE-YopE complex, with the conformation of SycE (gray) and the YopE Cb region corresponding to the crystal structure of SycE-YopE(Cb) (Birtalan, Phillips et al. 2002), and the conformation of the YopE RhoGAP domain corresponding to its crystal structure (Evdokimov, Tropea et al. 2002); other portions are depicted in random coil conformation.

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The abbreviations used are: TTS, type III secretion; Cb, chaperone-binding; S1, signal 1; TROSY, transverse relaxation optimized spectroscopy.

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III.

Mutations in the effector YopE that affect translocation but not chaperone binding

ABSTRACT

Pathogenic *Yersinia* species utilize the type III secretion system to deliver toxic effector proteins directly into the cytosol of host cells. The translocation of many effectors requires their prior association with specific chaperone proteins within the bacterium. In this study we investigate the hypothesis that chaperones target effectors to a TTS component required for translocation. A region of the YopE chaperone-binding domain adopts a β-hairpin configuration upon association with the chaperone SycE, and we find that a set of residues within this β-hairpin is required for YopE translocation but do not contribute to the YopE-SycE interaction. Rather, these solvent-accessible side chains project away from the complex and could be accessed by a binding partner. This structural motif is conserved among effectors, and may serve as a general signal through which chaperones identify their bound effectors as substrates for type III secretion.

INTRODUCTION

Type III secretion (TTS) systems are typically employed by Gram-negative bacteria to deliver virulence-related proteins, termed "effectors," directly into the cytosol of host cells (Ghosh 2004; Cornelis 2006; Galan and Wolf-Watz 2006). Syringe-like assemblies of > 20 proteins, termed "injectisomes," translocate a battery of effectors in support of diverse host-pathogen relationships. Translocation of many effectors requires their association with specific chaperone proteins in the bacterial cytosol. Consequently, chaperone-deficient bacterial strains have markedly reduced virulence compared to their wild-type counterparts (Rosqvist, Forsberg et al. 1990).

TTS chaperones bind one or few specific effectors. The well-characterized *Yersinia pseudotuberculosis* SycE-YopE chaperone-effector complex serves as a prototypical model. The ~ 50 residue chaperone-binding (Cb) domain of YopE is flanked by the carboxy-terminal RhoGAP effector domain and an amino terminal translocation signal. The first 15 amino acids, mRNA codons or both constitute a secretion signal, termed here S1, capable of driving the secretion of calmodulin-dependent adenylate cyclase into the extracellular space (Sory, Boland et al. 1995; Lloyd, Norman et al. 2001; Ramamurthi and Schneewind 2005). A second signal, termed S2, is hypothesized to reside within the Cb region of YopE. While S1 is sufficient for secretion, both

signals are required for translocation of YopE into PU5-1.8 macrophages (Sory, Boland et al. 1995). Translocation requires that the Cb region bind SycE, indicating that S2 does not lie in the effector alone, but rather requires association with the chaperone (Cheng, Anderson et al. 1997). The chaperone-effector complex is thus proposed to constitute a signal that is recognized by a bacterial component required for type III secretion. A prediction is that a set of residues that point outwards from the surface of the chaperone-effector complex is involved in interaction with this component, and therefore mutation of these residues should affect translocation. Comparison of the Cb domains of several TTS effectors reveals a conserved β-hairpin which is hypothesized to contribute to effector translocation (Birtalan, Phillips et al. 2002; Lilic, Vujanac et al. 2006). The stereochemical conservation and solvent-accessibility of Val23, Glu25 and Ser32 make these residues attractive candidates. These residues do not make contacts with SycE. Ser27 and Arg29 are likewise conserved, and interact with the chaperone through their side chains via hydrogen bonds. These interactions are likely to contribute to specificity and make only minor contributions to chaperone-effector affinity. In this study, we hypothesize that Val23, Glu25, Ser27, Arg29 and Ser32 contribute to effector translocation, and may constitute at least part of the proposed S2 signal, and we examine the role of these residues in the translocation of YopE.

Previous investigations of the translocation of TTS effectors into host cells have relied upon fusion constructs, including calmodulin-activated adenylate

cyclase, neomycin phosphotransferase, alkaline phosphatase, green fluorescent protein, ubiquitin, DHFR and TEM-1 beta-lactamase (Sory and Cornelis 1994; Anderson and Schneewind 1997; Jacobi, Roggenkamp et al. 1998; Feldman, Muller et al. 2002; Russmann, Kubori et al. 2002; Charpentier and Oswald 2004; Jaumouille, Francetic et al. 2008). While relatively straightforward to assay, the translocation of these reporters may be unrepresentative of native levels and the altered size, and stability of the constructs hinders comparisons to translocation of native protein. Here, we utilize selective lysis and quantitative Western blotting to investigate the role of this β-hairpin in YopE translocation, and we identify residues that play a role in effector translocation but do not contribute to association with SycE. These characteristics are consistent with these residues comprising a signal through which SycE targets YopE to the injectisome for translocation into host cells.

MATERIALS AND METHODS

Structural alignment

Structural alignments were performed in Pymol (DeLano 2002) using PDB files 1L2W, 1JYO, 1XKP, 2FM8 and 1TTW for YopE, SptP, YopN, SipA and YscM2, respectively. SptP residues 43-51 were aligned to YopE residues 25-32, YopN residues 33-40 were aligned to YopE residues 25-32, SipA residues 23-32 were aligned to YopE residues 27-33 and YscM2 residues 31-35 were aligned to YopE residues 29-33.

Strain and plasmid construction

The pIB virulence plasmid of serogroup III *Yersinia pseudotuberculosis* strain 126 (a gift from James Bliska) served as the PCR template from which all YopE and SycE constructs were derived (Table 3.1). Strain YPSB1 was also derived from this line, with YopE replaced by the pET28b (Novagen) kanamycin resistance gene by allelic exchange (Birtalan 2004). All vectors with a pKK designation are derivatives of pKK177-3, a 2.9-kb low-copy plasmid derived from pKK223-3 which carries the ampicillin resistance gene and the *tacl* promoter (Brosius and Holy 1984). The plasmids pKK-YopE, pKK-YopE-3Ala, and pKK-YopE-5Ala were generated by strand overlap extension PCR. These *yopE* variants were inserted into pKK-177-3, and transformed into YPSB1. All vectors

with a pcDNA designation are derivatives of pcDNA3.1+ (Invitrogen). pcDNA-YopE, pcDNA-YopE-3Ala, and pcDNA-YopE-5Ala were generated as described above, and inserted into pcDNA3.1+ using HindIII and XbaI restriction sites.

The plasmid pET28b-SycE-YopECb is a bicistronic derivative of pET28b consisting of nucleotides encoding residues 1-121 of *sycE* between NcoI and BamHI sites and 1-80 of YopE between NdeI and HindIII sites. pET28b-SycE-YopECb-3Ala was generated similarly, with YopE residues Val23, Glu25, and Ser32 mutated to alanine. The *yopE* gene of pET28b-SycE-YopECb-5Ala has Val23, Glu25, Ser27, Arg29 and Ser32 mutated to alanine.

Secretion assays

Yersinia strains used for secretion assays were cultured by shaking overnight at 26 ºC in 3 ml LB with 30 µg/ml kanamycin (for strains YP71 and YPSB1) or 30 µg/ml carbenicillin plus 30 µg/ml kanamycin (for strains YPDF1, YPDF2 and YPDF3). These were then sub-cultured into 10 ml LB at OD_{600}=0.1 with 20 mM sodium, 20 mM $MgCl_2$ and the antibiotics indicated above. In the case of a control wherein the secretion of Yops was inhibited, bacteria were sub-cultured in LB containing 2.5 mM $CaCl_2$. These cultures were grown with shaking for one hour at 26 ºC, at which time IPTG was added to a final concentration of 1 mM. Cultures were then grown an additional 3 hours at 37 ºC. Following this period, cells were harvested by centrifugation (10 minutes, 4 ºC, 4,000 x g). Cell pellets were washed twice with 1 ml 150mM NaCl, 50 mM

sodium phosphate, pH 8.0, and resuspended in SDS-PAGE sample buffer for analysis by Western blot of YopE expression (see below). Samples were taken from culture media and boiled with SDS-PAGE sample buffer for Western blot analysis of YopE secretion. Proteins from 8 ml of the remaining media from each culture were precipitated by addition of 1 ml 100% (w/v) trichloroacetic acid. Samples were rocked overnight at 4 ºC, and precipitated proteins were subsequently harvested by centrifugation (30 minutes, 4 ºC, 4,000 x g). Precipitates were washed twice with 1 ml acetone at 4 ºC, and residual acetone was removed by evaporation (15 minutes, 95 ºC). Samples were boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE.

Cell culture and infection assays

HeLa cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum (FBS), glutamine, streptomycin, and penicillin in a 5% CO_2 humidified incubator at 37 ºC. HeLa cells were split 18 hours prior to infection, and plated at a density of 2 x 10^6 cells per 10 cm dish. Two hours prior to infection, adherent cells were washed twice with PBS (37 ºC) and incubated in DMEM without FBS, penicillin, streptomycin, or glutamine. The proteosome inhibitory peptide MG-132 was added to a final concentration of 5 μM to inhibit ubiquitin-mediated YopE degradation (Ruckdeschel, Pfaffinger et al. 2006) and the actin polymerization inhibitor cytochalasin D was added to a final concentration of 1 μg/ml to ensure that actin disruption was equivalent in all

infections (Mejia, Bliska et al. 2008; Runco, Myrczek et al. 2008). The cells were then incubated for two hours at 37 ºC, 5% CO_2.

Two ml of Luria broth (LB) was inoculated with a single *Y. pseudotuberculosis* colony from an LB-agar plate. Bacteria were cultured overnight (approximately 15 hours) while shaking at 175 rpm, 26 ºC in LB supplemented with 30 µg/ml carbenicillin. Bacteria were then sub-cultured into 5 ml LB supplemented with 30 µg/ml carbenicillin and 2.5mM $CaCl_2$ to an OD_{600} of 0.1. After shaking for one hour at 175 rpm and 26 ºC, IPTG was added to a final concentration of 1 mM, and the cultures were then grown with shaking for an additional two hours at 37 ºC. One ml of cells was harvested by centrifugation (1 minute, 6,000 x g), and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Cellgro, 10-013-CV), supplemented with 1 µg/ml cytochalasin D (Biomol, T-109) and 5µM MG-132 (Calbiochem, 474790), to an OD_{600} of 1.0. A 75 µl sample of resuspended cells was collected and diluted to 200 µl with SDS-PAGE sample loading buffer for analysis of expression by Western blot. A 90 µl sample of bacteria was added to HeLa cells, producing an MOI of 1:50 (2×10^6 HeLa cells: 1×10^8 bacteria). Cells were infected for 150 minutes at 37 ºC, 5%CO_2. Non-adherent cells were removed with three 15 ml washes of cold (4 ºC) PBS, and following the aspiration of the last wash, papain was added to digest extracellular proteins (150 µl from a 0.25 mg/ml papain stock in PBS with 2.0 mM DTT). After 30 seconds, 140 µl of the papain solution was removed, leaving a thin layer of papain coating the HeLa monolayer. This

digestion proceeded for 20 minutes at room temperature, and was stopped by the addition of E-64 (90 µl from a 10 µg/ml stock). The cells were incubated in E-64 for five minutes before the addition of 400 µl lysis buffer (1% Triton-X 100, 10% glycerol, 150 mM NaCl, 10 mM Tris pH 8.0), which remained on the cells for five minutes. Cell lysates were collected by scraping and clarified by centrifugation (10 minutes, 16,000 x g, 4 ºC). Clarified lysate supernatants were filtered through a 0.22 µm cutoff membrane to remove intact cells and debris, and total protein content was quantified by a Lowry assay (Bio-RAD DC-protein detection kit) using a 96-well plate reader. Lysate volumes were adjusted with lysis buffer to make protein concentrations equivalent among samples; typical concentrations were one to two mg/ml as determined by comparison to a BSA standard. SDS-page sample buffer (4X) was added to the samples, which were then boiled for 10 minutes. Samples were centrifuged for 1 minute at 16,000 x g (room temperature), and were analyzed by Western blotting as described below.

Western Blotting

Samples were resolved by 12% SDS-PAGE and transferred to a PVDF membrane (Amersham #RPN303F, 1.8 mA/cm^2, 250 watts, 25 minutes). Membranes were blocked with 15 ml 5% milk in TBST (0.1% Tween-20, 50 mM Tris pH, 8.0, 150 mM NaCl) for 12 hours at 4 ºC. Twenty ml of primary antibody diluted in 5% milk (in TBST) were added (anti-YopE at a dilution of 1:4,000 and anti-tubulin at a dilution of 1:2,000), and incubated for 50 minutes at 25 ºC.

Membranes were washed 8 times with 30 ml TBST (3 minutes per wash), and 25 ml secondary antibody (HRP-conjugated goat anti-rabbit, Santa Cruz #sc-2004, diluted 1:30,000 in TBST) was added for 30 minutes at 25 ºC. Membranes were washed 8 times with 30 ml TBST (3 minutes per wash), and immunodetection was carried out on X-ray film with ECL plus (Amersham #RPN2132 and #RPN3114K) according to the manufacturer's instructions. Densitometry of X-ray film was performed using ImageJ (Abramoff 2004).

SycE-YopE(Cb) expression and purification

Truncations of *Yersinia pseudotuberculosis* SycE and YopE (including wild-type and the 3Ala and 5Ala YopE variants) were expressed and purified as described previously (Birtalan 2004), generating SycE 1-121 and YopE 1-80. Briefly, coexpression from pET28b was induced with 1 mM IPTG, and cells were grown at room temperature for three hours. The cells were lysed by sonication, and nucleic acids were removed from the clarified lysate by precipitation with 0.5% polyethyleneimine (PEI), and SycE-YopE(Cb) was precipitated with 50% saturated $(NH_4)_2SO_4$. The complex was further purified by anion-exchange chromatography (Poros HQ/M), cation-exchange chromatography (Poros HS/M) and size-exclusion chromatography (Superdex 75).

Guanidine denaturation

Wild-type, 3Ala or 5Ala SycE-YopE(Cb), or SycE-his was concentrated to 1.55 μM in 100 mM NaCl and 10 mM phosphate buffer, pH 8.0. Denaturation was assayed on an Aviv 202 circular dichroism spectrometer at 222 nm. Guanidine was increased by automated titration (Hamilton Microlab 500 titrator), with stepwise addition of 5.7 M guanidine with 1.55 μM protein, 100 mM NaCl and 10 mM phosphate buffer, pH 8.0. Guanidine concentration was determined by refractive index. The sample was stirred for 10 minutes between titration, and spectroscopic changes were complete within this interval (data not shown). Each titration step was measure for 15 seconds at 25 ºC.

Spectroscopic calculations

Spectroscopic data were analyzed essentially as described previously (Birtalan 2004). Observed circular dichroism elipticity (θ_{obs}) measurements were corrected for concentration by conversion to mean residue molar elipticity ([θ], deg cm^2/dmol):

$$[\theta] = \frac{\theta_{obs}}{10\,lcn} \quad \text{(equation 1)}$$

where θ_{obs} is observed elipticity in millidegrees, l is the sample path length in cm, c is the molar protein concentration and n is the sum of the number of residues in the SycE homodimer or SycE-YopE(Cb) heterotrimer. Molar elipticity was then plotted as a function of guanidine chloride concentration, and changes in optical

properties were compared by normalizing each transition curve to the apparent fraction of the unfolded form (F_{app}):

$$\mathrm{Fapp} = \frac{([\theta]_{obs} - [\theta]_{nat})}{([[\theta]_{unf} - [\theta]_{nat})} \quad \text{(equation 2)}$$

where $[\theta]_{obs}$ is the observed mean residue molar elipticity at a given guanidine concentration, and $[\theta]_{nat}$ and $[\theta]_{unf}$ are the observed values for the native and unfolded forms, respectively, at the same protein concentration (Bae, Chou et al. 1988). A linear dependence of $[\theta]_{obs}$ on the denaturant concentration was observed in both the native and unfolded base line regions, and linear extrapolations from these baselines were made to obtain estimates of $[\theta]_{nat}$ and $[\theta]_{unf}$ in the transition region.

We calculated dissociation constant for the binding of SycE to YopE(Cb) (wild-type, 3Ala and 5Ala) by comparing the guanidine-dependant stability of each construct to that of SycE alone. As described previously (Birtalan 2004), we used Mathematica (Wolfram Research) scripts developed by Dr. Terrance Oas (Duke University) to determine the unfolding transition midpoint (C_{mid}) and guanidine-dependence (m) to the apparent fraction of the unfolded form (F_{app}) for the SycE homodimer:

$$F_{app} = \frac{F_d P_{conc}}{F_d P_{conc} + 2e^{-\frac{-(C_{mid} + Den)m}{RT}} F_d^{\,2} P_{conc}^{\,2}} \quad \text{(equation 3)}$$

Where P_{conc} is protein concentration, R is the gas constant, 1.9872 X 10^{-3}, and T is the temperature in Kelvin. Equation four applies these values of c_{mid} and m from the SycE homodimer to unfolding data of SycE-YopE(Cb) to determine the association constant, K_A, for the complex. The apparent fraction of denatured protein, F_{app}, is related to the equilibrium constant of unfolding, K_F, the protein concentration, P_{conc}, and K_A by:

$$F_{app} = \frac{F_d P_{conc}}{F_d P_{conc} + 2F_d{}^2 K_F P_{conc}{}^2 + \frac{2F_d{}^2 K_A K_F P_{conc}{}^3}{1 + F_d{}^2 K_A K_F P_{conc}{}^2}} \quad \text{(equation 4)}$$

where K_F is related to C_{mid} by:

$$K_F = e^{\frac{-(m(Den - C_{mid}))}{RT}} \quad \text{(equation 5)}$$

The K_D can then be calculated as:

$$K_D = \frac{1}{K_A} \text{(equation 6).}$$

RESULTS

Identification of conserved effector residues

The chaperone SycE promotes the ordering of the Cb domain of YopE (chapter two). We compared the structures of chaperone-bound TTS effectors in order to identify residues that might target the chaperone-effector complex to another TTS component. Fragments of *Y. pseudotuberculosis* YopE, *Y. pestis* YopN, *Y. pestis* YscM2, *S. typhimurium* SptP, and *S. typhimurium* SipA have been crystallized in complex with their respective chaperones (Stebbins and Galan 2001; Birtalan, Phillips et al. 2002; Phan, Tropea et al. 2004; Schubot, Jackson et al. 2005; Lilic, Vujanac et al. 2006). Alignment of these effectors demonstrates the horseshoe-shaped path of these proteins as they wrap around their chaperones (Fig 3.1A,B). These effectors align remarkably well at the amino-termini of the Cb domains (Lilic, Vujanac et al. 2006). In YopE, this region contains a hairpin formed by the first two β-strands (β1 and β2). Residues from the β2 strand of YopE form hydrogen bonds with the outer edge of a SycE β-sheet, and the same configuration is found in SicP-SptP(Cb) and SycN-YopN(Cb) (Stebbins and Galan 2001; Birtalan, Phillips et al. 2002; Schubot, Jackson et al. 2005). YscM2(Cb) forms a β-strand which binds the chaperone SycH in much the same way as YopE(Cb) β-2 binds SycE (Phan, Tropea et al. 2004). Residues preceding this YscM2 β-strand were absent from the crystallized effector fragment. SipA

was also found to form an intermolecular β-sheet with the chaperone InvB, and aligns well with the YopE(Cb) β2 (Lilic, Vujanac et al. 2006). Unlike YopE, this SipA β-strand is preceded by a short α-helix (α1). In spite of this difference, the α1-β1 region of SipA produced a distribution of hydrophobicity remarkably similar to that of YopE, SptP, YopN and YscM2 (Fig. 3.1D) (Lilic, Vujanac et al. 2006).

Three hydrophobic YopE residues (V23, M26 and V31) align with hydrophobic residues of superimposed effectors (Fig 3.1C,D), and sequence similarity suggests that this distribution of hydrophobic residues is utilized widely by TTS substrates as a conserved means of associating with chaperones (Schesser, Frithz-Lindsten et al. 1996; Lilic, Vujanac et al. 2006). Indeed, YopE M26 and V31 make hydrophobic contacts with SycE. Although V23 does not contact SycE, aligned residues indicate that this position is also occupied with hydrophobic residues in SptP and YopN.

We sought to examine the possibility that residues with the YopE Cb region constitute an S2 translocation-targeting motif. We hypothesized that residues that constitute such a motif might be conserved, and we sought to identify residues within the conserved β-hairpin that are chemically similar among TTS effectors. Residues aligning with YopE S30 and Q33 showed little conservation (Fig. 3.1D). YopE M26 and V31 bind the effector to SycE through hydrophobic interactions and are unlikely to comprise the S2 signal. Of the

remaining residues within the YopE β1-β2 hairpin, V23, E25, S72, R29 and S32 are attractive candidates for the S2 signal. V23, E25 and S32 do not interact with SycE, and their solvent-exposed side chains extend away from the complex. The chemical properties of these residues are conserved among the effectors whose chaperone-bound structures have been determined (Fig. 3.1). Therefore, a triple mutation was generated (YopE-3Ala) which converted these residues to alanine (Fig. 3.1B). Residues which align with Ser27 and Arg29 are without exception hydrophilic (Fig 3.1D). These YopE residues form intermolecular hydrogen bonds with SycE, interactions which are likely to contribute to specificity rather than binding energy. Since disruption of these residues was unlikely to disrupt the chaperone-effector interaction, we generated a second YopE mutant (YopE-5Ala) in which converted S27 and R29 in addition to V23, E25, and S32 were substituted with alanine (Fig. 3.1B).

SycE-YopE denaturation.

We sought to determined whether substitution of V23, E25, S72, R29 and S32 to alanine disrupted the chaperone-effector interaction. We first examined whether substitution of V23A, E25A, and S32A to alanine disrupted the chaperone-effector interaction. We therefore subjected purified SycE-YopE(Cb) complexes to guanidine denaturation studies monitored by circular dichroism spectroscopy at 222 nm. The primary contributor to far-UV CD signal at 222 nm

is α-helical structure. It has been demonstrated that the guanidine concentration midpoint (C_{mid}) for unfolding is 2.4 M for SycE and 2.8 M for the SycE-YopE(Cb) fragment (Birtalan, Phillips et al. 2002). The difference in C_{mid} reflects the SycE-YopE(Cb) binding energy, which is calculated to correspond to 8.2 kcal/mol when extrapolated to 0 M guanidine. The chaperone-binding region is independent from the remainder of YopE (chapter two) (Birtalan, Phillips et al. 2002), and we therefore eliminated contributions from the RhoGAP domain by utilizing truncated SycE-YopE complexes consisting of the first 122 residues of SycE and the first 80 residues of YopE, which is termed "YopE(Cb)" here (Birtalan, Phillips et al. 2002).

Chemical denaturation profiles were measured for SycE and SycE-YopE(Cb) complexes (WT, 3Ala and 5Ala). Guanidine-induced unfolding of SycE and SycE-YopE(Cb) were found to be reversible (data not shown). Using concentrations of SycE-YopE(Cb) that differ by 25-fold, we observed that the midpoint of the unfolding transition depends on the concentration of the complex, as would be expected for a bimolecular binding reaction (Fig. 3.2).

All denaturation curves were sigmoidal with a single sharp transition and no inflections. These data fit well to a two-state model with no intermediates detected for SycE-YopE(Cb) (Fig. 3.2). The interaction of SycE with wild-type YopE(Cb) was relatively tight, with a calculated K_D of 21 nM ± 4 nM. We calculated a K_D of 21 nM ± 2 nM for the SycE-YopE(Cb)-3Ala interaction, demonstrating that mutations substituting Val23, Glu25 and Ser32 with alanine

did not affect the affinity of the chaperone-effector interaction. These calculations are consistent with the superposition of the SycE-YopE(Cb) and SycE-YopE(Cb)-3Ala unfolding reactions (fig. 3.3). Additional substitutions of Ser27 and Arg29 with alanines were found to disrupt binding very slightly, with a K_D of 36 nM ± 3 nM for SycE-YopE(Cb)-5Ala. Differences between KD values measure for SycE-YopE(Cb) and SycE-YopE(Cb)-5Ala were significant (ANOVA, P=0.03)

YopE secretion and translocation

The term "translocation" describes the act of delivering effectors into targeted host cells, and "secretion" is used to describe a phenomenon wherein effectors are released into the extracellular media. Chaperones are required for effector translocation, but are dispensable for secretion. The proposed S2 translocation-targeting signal is therefore thought to contribute to translocation, but not secretion. To determine if the YopE β1-β2 hairpin is required for secretion, we compared the secretion of wild-type YopE to YopE-3Ala and YopE-5Ala. We found that these sets of mutations had no effect on YopE secretion (Fig 3.4).

A HeLa infection assay was carried out to determine if residues within the YopE β1- β2 hairpin play a role in YopE translocation. *Y. pseudotuberculosis* lacking *yopE* was transformed with a low-copy plasmid encoding either wild-type

YopE, YopE-3Ala, or YopE-5Ala (Table 3.1). HeLa cells were infected with these *Yersinia* strains and selectively lysed in Triton-X 100. YopE was found to be translocated with comparable efficiency from wild-type *Yersinia* (YP126) and strains constitutively expressing YopE from the low-copy plasmid pKK177-3 (YPDF1, see methods and Table 3.1), indicating that expression from pKK177-3 did not affect YopE translocation (Fig. 3.5A,B). YopE-3Ala and YopE-5Ala were significantly deficient in translocation as compared with wild-type YopE (Table 3.2) (Fig. 3.5A,B).

As a negative control for translocation, we utilized *Yersinia* deficient in YopB, a protein necessary for forming pores in targeted host cells through which effectors transit. This strain is capable of secreting but not translocating Yops (Hakansson, Schesser et al. 1996; Nordfelth and Wolf-Watz 2001). A small amount of YopE was detected in lysates of HeLa cells infected with *ΔyopB Yersinia* (Fig. 3.5A,B). In contrast to previous results (Nordfelth and Wolf-Watz 2001), protease treatment (papain and proteinase K) did not reduce the amount of YopE detected lysates of HeLa cells infected by Δ*yopB Y. pseduotuberculosis* (data not shown). We speculated that bacterial lysis might be releasing YopE during HeLa infection assays. However, anti-YopE Western blots reveal that no YopE was released by bacteria incubated for three hours in lysis buffer (data not shown). Anti-SycE western blots demonstrate that SycE, which remains within the bacterium (Frithz-Lindsten, Rosqvist et al. 1995), was not present in lysates of infected HeLa cells (data not shown). We therefore conclude that bacterial lysis

does not release YopE during in the course of a HeLa cell infection. Next, we sought to determine whether the TTS system was exporting YopE from the *ΔyopB* strain. We utilized strain YP71, which harbors an unmapped Tn5 transposon and is unable to secrete Yops (Bolin and Wolf-Watz 1984). YP71 expressed but did not export any detectable YopE in secretion or translocation assays, indicating that the export of YopE from *ΔyopB Yersinia* was TTS-dependent (Fig. 3.6).

DISCUSSION

Chaperone-effector association is requisite for effector translocation, and we propose that this interaction targets the effector to a component of the TTS system. The interaction orders the effector Cb region, suggesting that chaperones might template the formation of a targeting motif (chapter two) (Birtalan, Phillips et al. 2002). We sought to identify the YopE residues that comprise such a targeting signal and focused our investigation on the conserved β-hairpin stretching from Val23 to Ser32 within the YopE Cb region (Birtalan, Phillips et al. 2002). We tested two groups of either three or five solvent-accessible YopE residues that were chemically conserved in other effectors: Val23, Glu25, Ser27, Arg29 and Ser32 (Fig. 3.1). To examine the role of these five residues in translocation, we substituted these as a set of three (Val23, Glu25, and Ser32) or five (Val23, Glu25, Ser27, Arg29 and Ser32) with alanine and assayed the ability of *Yersinia* to translocate YopE into HeLa cells. We found that YopE-3Ala and YopE-5Ala were translocated at low levels, equivalent to the level of background translocation by the translocation-defective Δ*yopB* strain of *Y. pseudotuberculosis*. The inability of this strain to form pores in host cells provides a quantification of YopE that is exported by the TTS injectisome but does not enter targeted cells. The absence of translocated YopE from YP71, which cannot secrete or translocate Yops (Bolin and Wolf-Watz 1984), indicates

that YopE detected from lysates of HeLa cells infected with Δ*yopB Yersinia* was TTS-dependent. This does not necessarily indicate that YopE translocated from Δ*yopB Yersinia* was internalized into HeLa cells. It is noteworthy that this YopE was resistant to proteolysis by both papain and trypsin, and protection from proteolysis might have been due to association with cell membranes or the formation of detergent-soluble YopE aggregates that are resistant to protease treatment. The hypothesis that extracellular YopE adheres to HeLa membranes is supported by the observation that hydrophobic residues within the YopE Cb region (residues 54-75) localize transfected YopE to the membrane of HeLa cells (Krall, Zhang et al. 2003).

We investigated the possibility that a phenomenon other than impaired translocation reduced the quantity of mutated YopE detected from infected HeLa cells. None of the mutations studied had a discernable effect on YopE expression (Fig. 3.5A,C). We set out to determine if mutations in YopE cause any post-translocation effects (such as protein degradation or precipitation) which might appear to be differential translocation. YopE has been shown to be degraded by the ubiquitin-proteosome pathway, and we utilized the proteosome inhibitor MG-132 to minimize YopE degradation (Ruckdeschel, Pfaffinger et al. 2006). None of the residues mutated in this study are likely to disrupt ubiquitin-mediated proteolysis. Nonetheless, we are investigating the stability of wild-type YopE,

YopE-3Ala and YopE-5Ala introduced into HeLa cells by transfection (see Appendix).

We also address the possibility that mutations of these residues disrupt translocation by disrupting the chaperone-effector interaction. Residues within the YopE β1-β2 hairpin confer association with SycE via hydrogen bonds and hydrophobic interactions (Birtalan, Phillips et al. 2002). We show that mutation of Ser27 and Arg29 to alanine only slightly reduces the tight chaperone-effector interaction in the background of the other three substitutions, and these mutations do not prevent YopE from associating with SycE (Fig. 3.3). Val23, Glu25, and Ser32 are known to not bind SycE, and mutation of these residues did not alter the SycE-YopE affinity. Thus, the defective translocation exhibited by YopE-3Ala and YopE-5Ala cannot be attributed to an inability to associate with SycE. The residues of the YopE β1-β2 hairpin contribute to translocation in a manner consistent with the hypothesis that this region constitutes the S2 signaling motif.

Much effort has been applied to identifying the chaperone-templated signal's target. Chaperones are found solely in the bacterial cytosol (Frithz-Lindsten, Rosqvist et al. 1995), indicating that the role of the chaperone is likely fulfilled prior to the effector's passage through the base of the type III secretion apparatus. Probable receptors thus include the inner-membrane TTS apparatus components, especially those with predicted globular cytoplasm domains such as YscU, YscV, and YscR (Ghosh 2004). Furthermore, the *Salmonella* ATPase

InvC has been shown to bind the effector SptP in complex with the chaperone SicP (Akeda and Galan 2005). However, evidence of an interaction between the *Yersinia* ATPase YscN and SycE-YopE complexes has not been forthcoming (chapter two). It remains possible that the mechanism of effector recognition varies between species.

Chaperones of the type III secretion system are critical for the efficient translocation of the effectors they bind. We have previously demonstrated that association with SycE produces order in the YopE Cb region, and templates the formation of a conserved β-hairpin. Here we identify residues within this structural feature that are critical for YopE translocation. Disruption of residues within this β-hairpin impairs translocation but not secretion. This observation parallels the dependence of effectors on chaperones for translocation into host cells, but not secretion into culture media. Together, these data support the hypothesis that the YopE β1-β2 hairpin serves as an S2 targeting motif formed upon association with SycE.

FIGURES AND TABLES

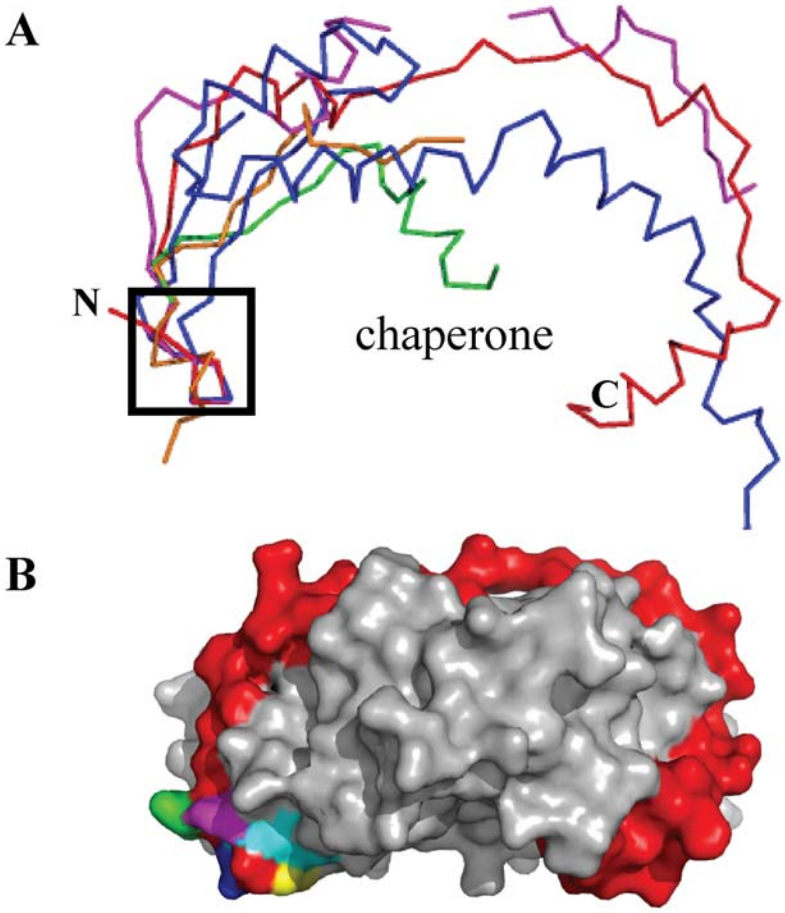

Figure 3.1. Chemical similarity within chaperone-binding domains.
A. Superimposed Cα traces depict *Yersinia pseudotuberculosis* YopE (red), *Salmonella typhimurium* SptP (blue), *Yersinia pestis* YopN (magenta), *Salmonella typhimurium* SipA (orange) and *Yersinia pestis* YscM2 (green). Amino and cayboxy termini of YopE(Cb) are indicated with an "N" and "C." Alignments were based upon amino-terminal residues (see methods), indicated by a box. Using YopE as a reference, the amino-termini of SptP, YopN, SipA and YscM2 aligned with RMSD values of 0.844 Å (eight Cα positions), 0.363 Å (seven Cα positions), 0.939 Å (nine Cα positions) and 0.620 Å (four Cα positions), respectively. All effectors were crystallized in complex with chaperones, which are represented by the label "chaperone" for simplicity.
B. Molecular surface representation of the SycE-YopE(Cb) complex. The SycE homodimer is depicted in gray. YopE residues are red, with the exception of V23 (green), E25 (blue), S27 (yellow), R29 (cyan) and S32 (magenta)

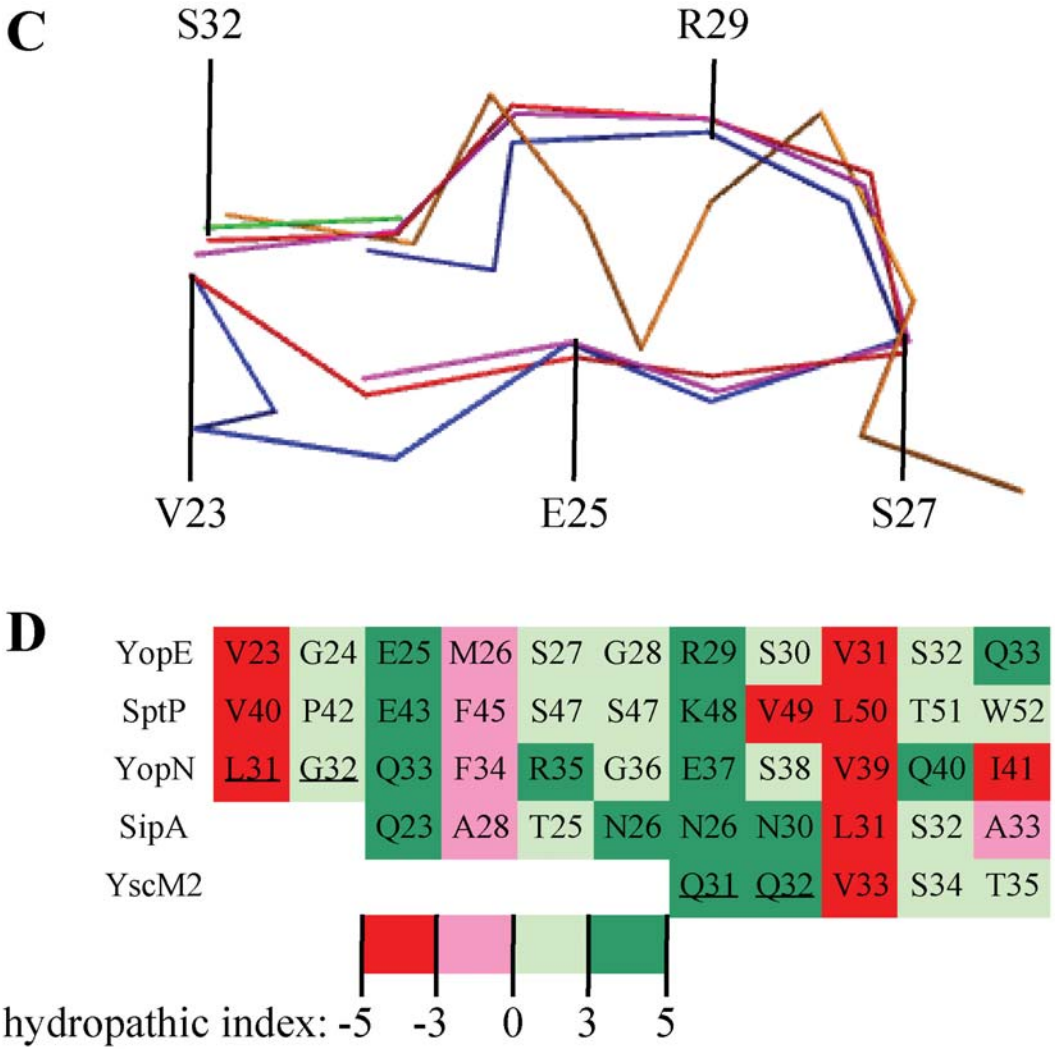

Figure 3.1 continued.
C. Cα-traces of superimposed effectors within the boxed region of **A.** Alignment and color is as described in **A**. Labels indicate YopE residues.
D. Residues shown in **C** are aligned based on three-dimensional position. Underlined residues were absent from crystallographic models and are proposed to occupy the indicated positions based on sequence extrapolation. Colorimetric hydropathy scale was derived from previously reported amino acid hydrophobicity values (Kyte and Doolittle 1982).

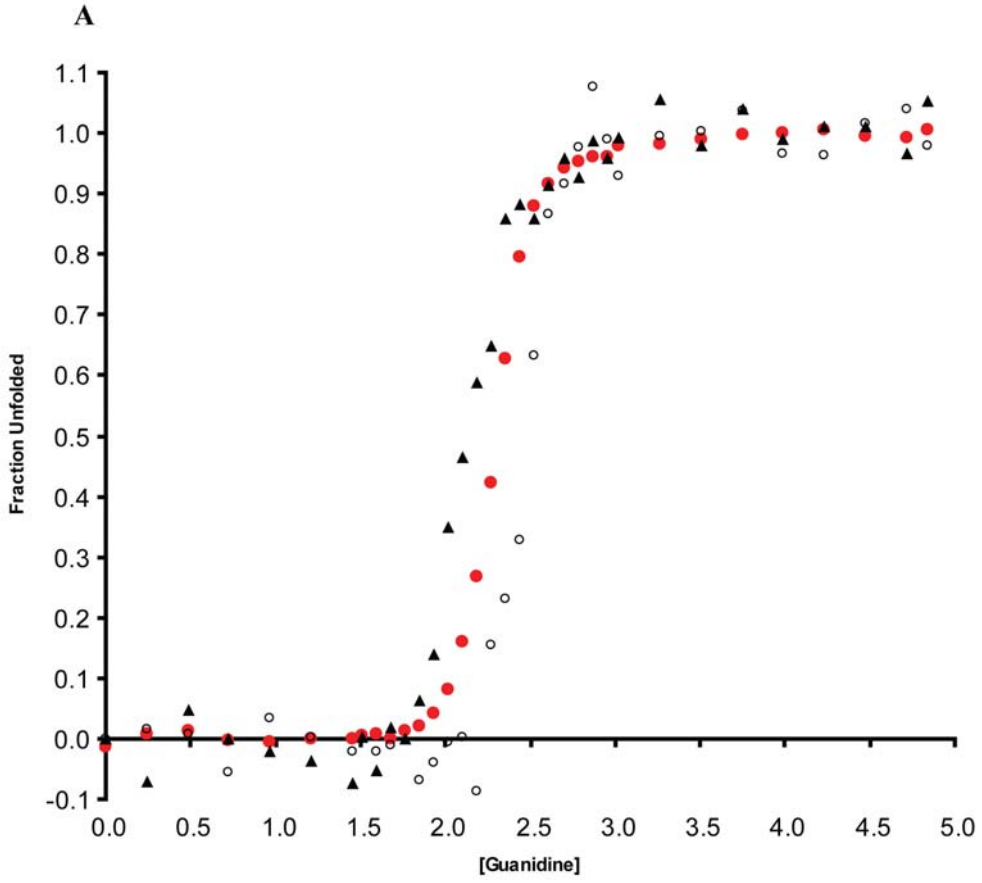

Figure 3.2. Effect of protein concentration on SycE-YopE(Cb) chemical denaturation.

The dependence of the apparent fraction of unfolded protein (F_{app}) is plotted as a function of guanidine concentration.

A. Wild-type SycE-YopE(Cb) complexes unfolded at 0.31 μM, 1.55 μM and 7.75 μM, represented by black triangles, red circles and open black circles, respectively.

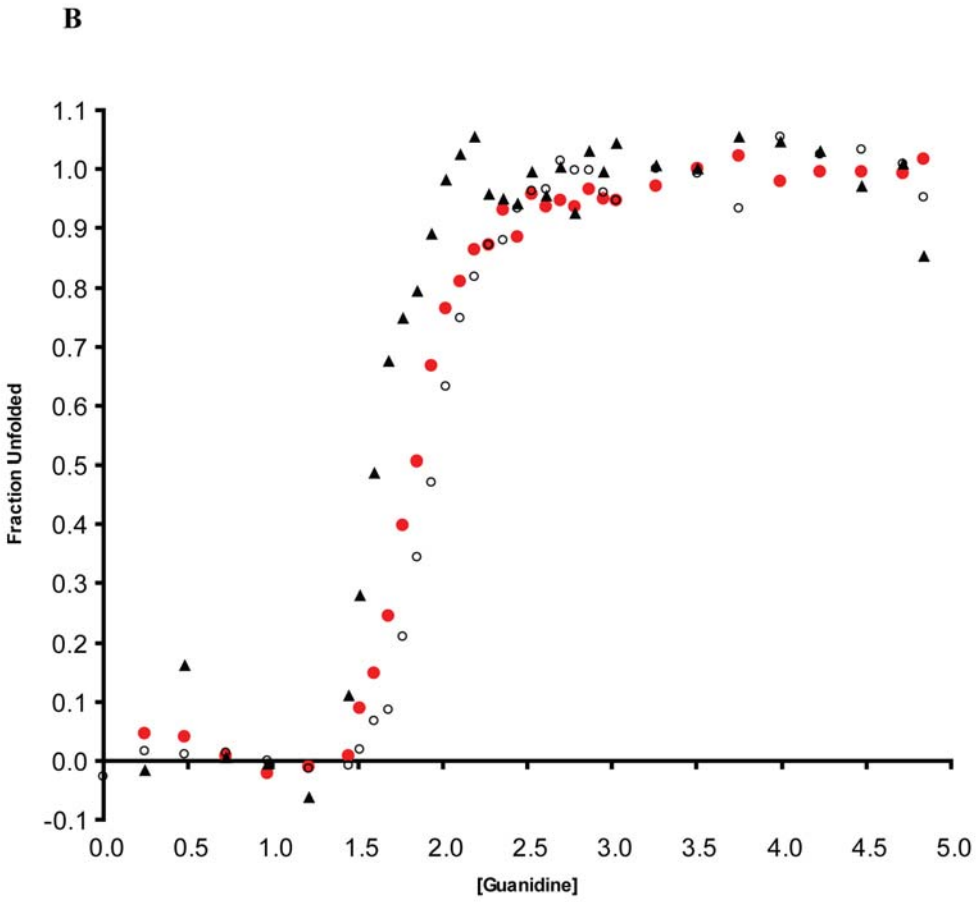

Figure 3.2 continued.
B. SycE homodimers are unfolded as described in **3.4A**. Black triangles, red circles and open black circles denote SycE unfolding using protein concentrations of 0.31 μM, 1.55 μM and 7.75 μM respectively.

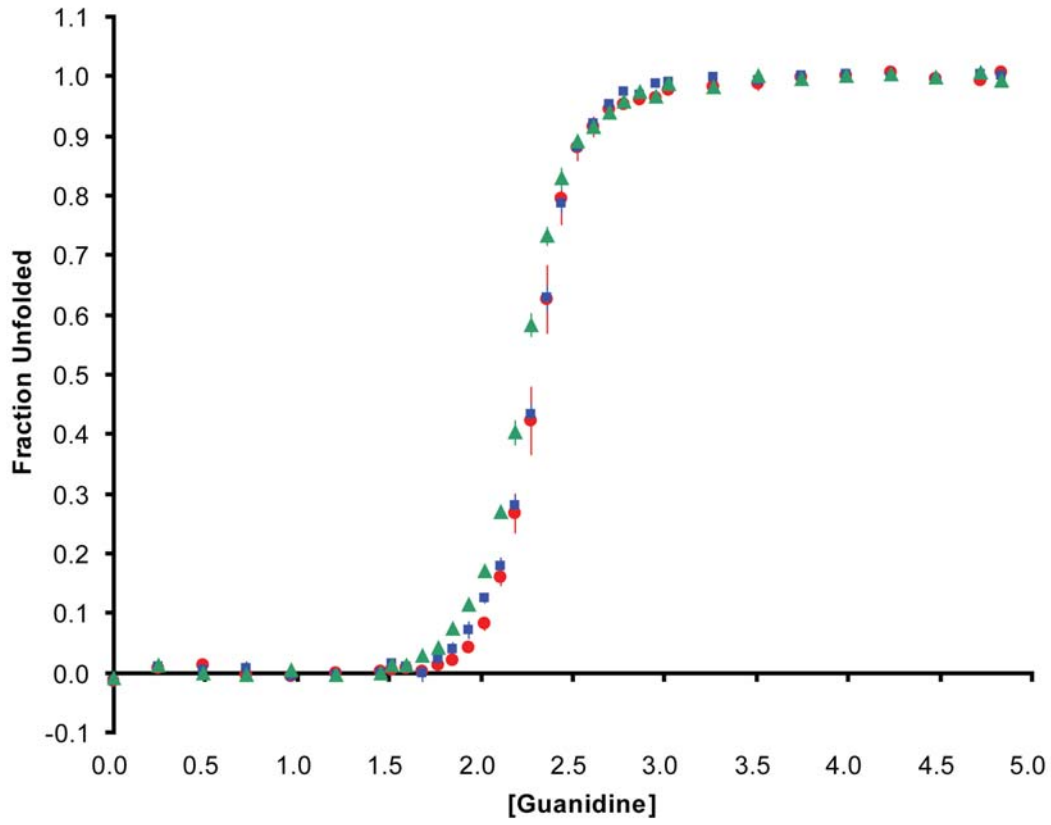

Figure 3.3. Effect of mutations on SycE-YopE(Cb) chemical denaturation. Dependence of the apparent fraction of unfolded protein (F_{app}) is plotted as a function of guanidine concentration. Red circles, blue squares and green triangles represent wild-type, 3Ala and 5Ala versions of the SycE-YopE(Cb) complex, respectively. Data were averaged from at least three independent experiments, and bars represent standard error of the mean.

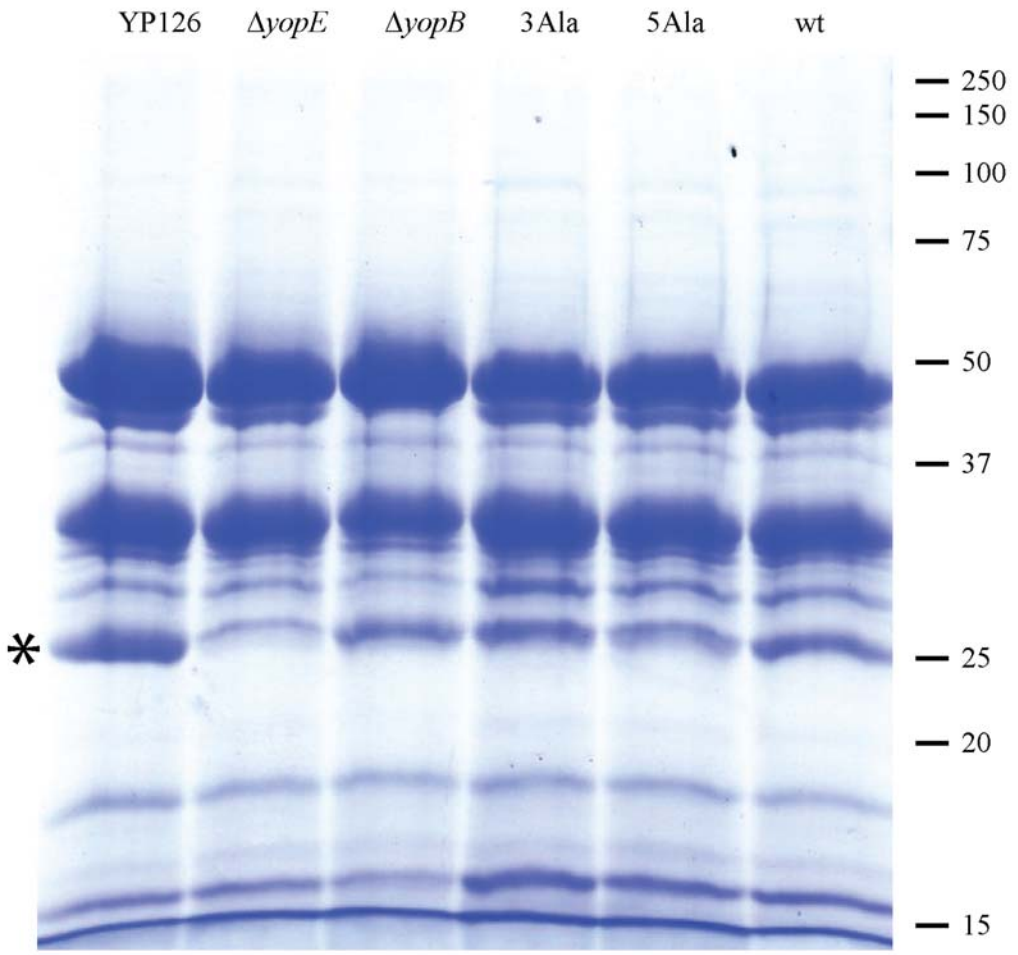

Figure 3.4. Mutations of V23, E25, S27, R29, and S32 do not disrupt YopE secretion.
SDS-PAGE of TCA precipitated supernatants from *Y. pseudotuberculosis* cultures in secretion-inducing conditions. YopE (marked by an asterisk) is secreted comparably by all strains with the exception of Δ*yopE* (*yopE::kan*). Wild-type YopE, YopE-3Ala, and YopE-5Ala YopE (respectively "wt," "3Ala" and "5Ala") were expressed from the exogenous low-copy pKK plasmid. *Y. pseudotuberculosis* strain 126 is designated "YP126." Molecular weights are indicated in kD.

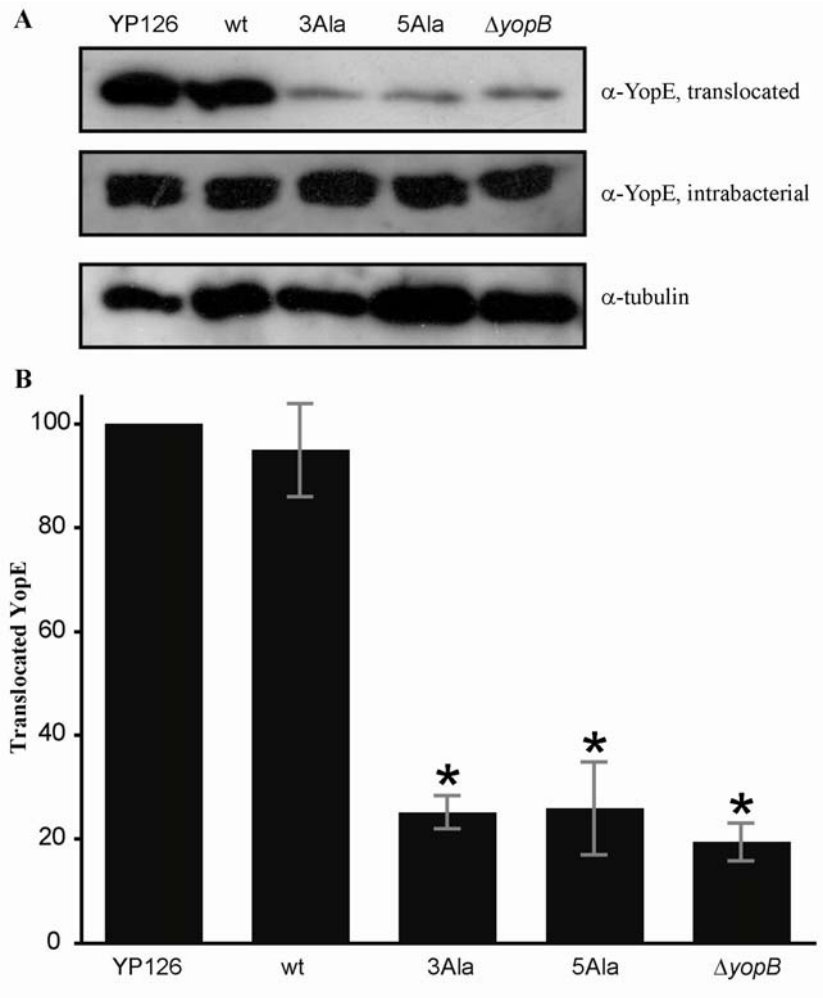

Figure 3.5. Mutations of V23, E25, S27, R29, and S32 disrupt YopE translocation.

A. YopE translocated into *Y. pseudotuberculosis*-infected HeLa cells. HeLa cells were infected with *Yersinia* and selectively lysed by detergent incubation. Translocated YopE was detected from HeLa lysates by α-YopE Western blot. *Y. pseudotuberculosis* strain 126 is designated "YP126" here. Intrabacterial YopE was detected by α-YopE Western blot from *Yersinia* lysates prior to infection. Tubulin was detected by α-tubulin Western blot from HeLa lysates after infection. Typical results are shown. Experiments were repeated three times, with each sample in duplicate or triplicate.

B. Densitometry of translocated YopE carried out from Western blots of each experiment. Results were normalized to the level of YopE translocated from the wild-type strain. Densitometry was within a linear range of measurement, and bars report standard error of the mean. An asterisk indicates a significant difference from YP126, e.g., $F > F_{crit}$ and $P < 0.01$.

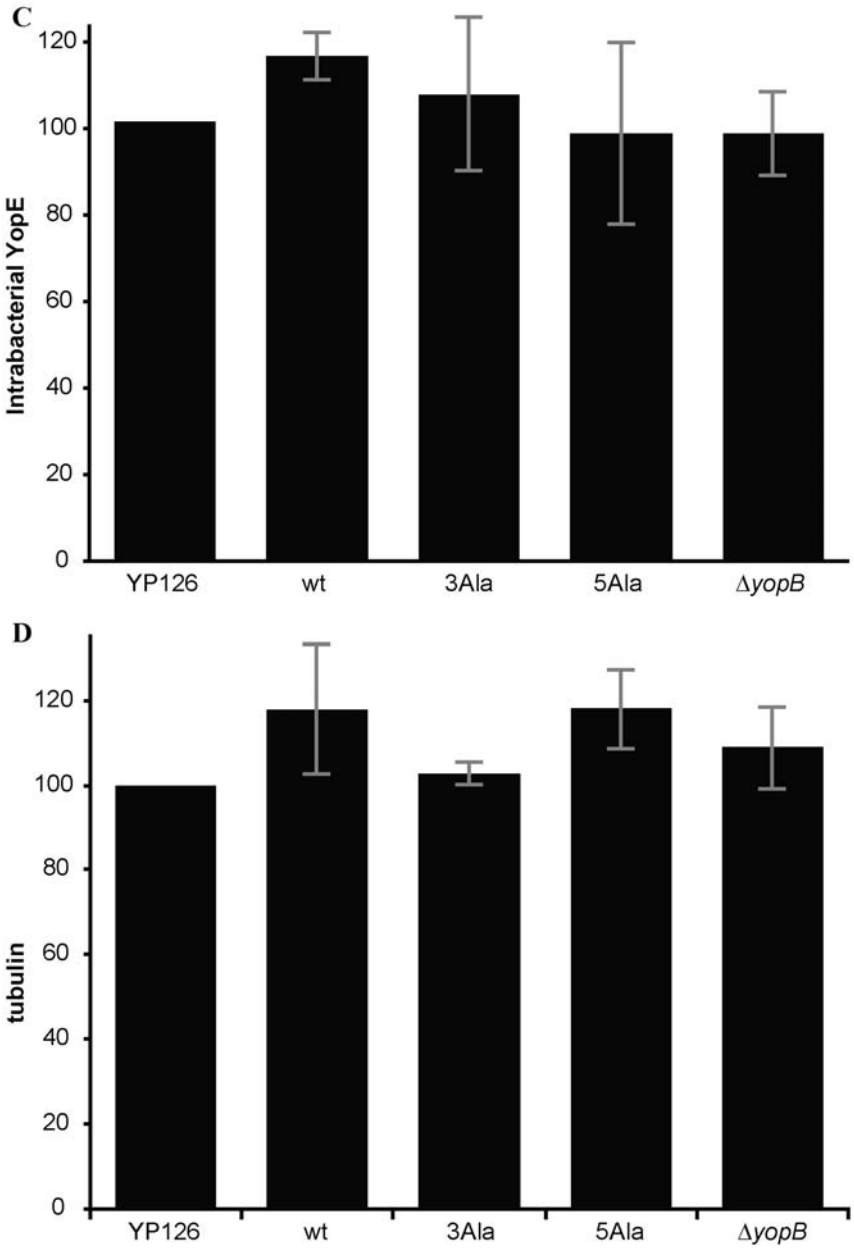

Figure 3.5 continued.
C. Densitometry of anti-YopE Western blots of bacterial lysates to examine YopE expression. Values were normalized as described in Fig. 3.5B.
D. Densitometry of anti-tubulin Western blots. Values were measured from HeLa cell lysates of each infection, and results were normalized to the level of tubulin detected from HeLa cells infected by the *Yersinia* strain YP126.

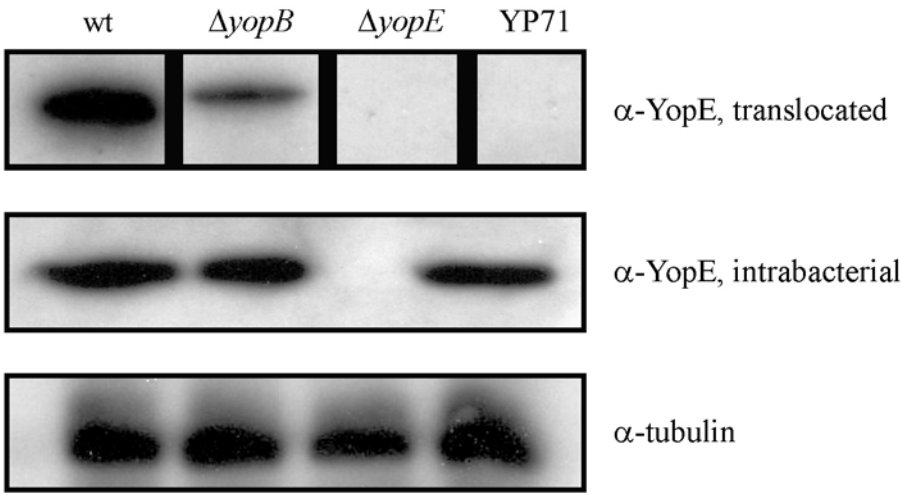

Figure 3.6. YopE detected from lysates infected with Δ*yopB Y. pseudotuberculosis* is TTS-dependent.
YopE and tubulin, as detected by immunoblots. HeLa cells were infected for three hours at an MOI of 1:50 (HeLa: *Y. pseudotuberculosis*), and selectively lysed by detergent incubation. Wild-type YopE, (wt) was expressed exogenously from pKK. Clarified lysates were normalized based on total protein concentration, and translocated YopE, intrabacterial YopE, and endogenous tubulin were detected by immunoblots.

Table 3.1. Plasmids and bacterial strains.
All plasmids and strains used in this study are described. All *Yersinia* strains are derivatives of *Y. pseudotuberculosis* strain 126.

Plasmid	**Characteristics**	**Reference**
pKK-YopE	Wild-type *yopE* in pKK177-3	This work
pKK-YopE-3Ala	*yopE* (V23A, E25A, S32A) in pKK177-3	This work
pKK-YopE-5Ala	*yopE* (V23A, E25A,S27A, R29A, S32A) in pKK177-3	This work
pcDNA-YopE	Wild-type *yopE* in pcDNA3.1+	This work
pcDNA-YopE-3Ala	*yopE* (V23A, E25A, S32A) in pcDNA3.1+	This work
pcDNA-YopE-5Ala	*yopE* (V23A, E25A, S27A, R29A, S32A) in pcDNA3.1+	This work
Pet28b-SycE-YopECb	*sycE* 1-121 and *yopE* 1-80 in Pet28b	This work
Pet28b-SycE-YopECb-3Ala	*sycE* 1-121 and *yopE* 1-80 (V23A, E25A, S32A) in Pet28b	This work
Pet28b-SycE-YopECb-5Ala	*sycE* 1-121 and *yopE* 1-80 (V23A, E25A,S27A, R29A, S32A) in Pet28b	This work
Pet28b-SycE-His	6Xhis-tagged *sycE* in Pet 28b	This work
Bacterial Strain	**Characteristics**	**Reference**
YP126	Wild-type *Yersinia pseudotuberculosis* 126, YPIII (P^+)	(Bolin, Norlander et al. 1982)
YPSB1	YP126*yopE::kan*	(Birtalan 2004)
YPDF1	YP126*yopE::kan* with pKK-YopE	This work
YPDF2	YP126*yopE::kan* with pKK-YopE-3Ala	This work
YPDF3	YP126*yopE::kan yopE* with pKK-YopE-5Ala	This work
YP18	YP126Δ*yopB* (deficient in YopB)	(Palmer, Hobbie et al. 1998)
YP71	YP126*ysc*::Tn5 (deficient in secretion of all Yops)	(Bolin and Wolf-Watz 1984)

Table 3.2. Single-factor ANOVA test applied to translocation data. Variance between densitometry data collected from translocation samples (**Fig. 3.3**) was analyzed for statistical significance by means of a single-factor ANOVA test in Microsoft Excel. F_{crit} was calculated with a rejection probability (α) of 0.05, and the null hypothesis is rejected if $F > F_{crit}$ and P< 0.01.

Strains compared	***F***	F_{crit}	**P-value**
Translocated YopE			
YP126, YPDF1	0.3226	7.7087	0.6004
YPDF2, YPDF3, YP18	0.3546	5.1433	0.7153
YPDF1, YPDF2, YPDF3, YP126-YP18	28.3098	4.0662	0.0001
Intrabacterial YopE			
YP126, YPDF1, YPDF2, YPDF3, YP18	0.3301	3.0556	0.8534
Tubulin			
YP126, YPDF1, YPDF2, YPDF3, YP18	0.8260	3.4781	0.5379

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IV.

Future directions

ABSTRACT

Pathogenic *Yersinia* spp. utilize a type III secretion system to translocate effector proteins into host cells during the course of an infection. Targeting signals are known to distinguish export substrates from the many proteins which remain within the bacterium. One signal resides within the first 15 effector codons or amino acids. Another targeting signal is proposed to be formed when specific chaperone proteins associate with effectors. Several interactions between effectors and components of the type III secretion machinery have been reported, but none have been shown to recognize either signal. Here we present preliminary results of investigations seeking to identify the receptor for these targeting signals. It seems probable that inner-membrane injectisome components participate in effector recognition, and I make recommendations for future studies.

INTRODUCTION

Many Gram-negative pathogens utilize a type III secretion (TTS) system to translocate effector proteins into host cells (Ghosh 2004; Cornelis 2006; Galan and Wolf-Watz 2006). *Yersinia* effector proteins are termed "Yops" and disrupt host signaling to disable phagocytosis, inhibit the inflammatory response and modulate cell death (Navarro, Alto et al. 2005). Yops and are exported from the bacterium though the syringe-like "injectisome," an apparatus composed of over 20 distinct proteins. Proteins destined for export through the injectisome are thought to utilize two distinct signals to distinguish themselves from the greater number of proteins which will remain within the bacterium (Ghosh 2004). Residues or codons 1-15 of most Yops constitute one signal, termed S1, capable of driving the secretion of many reporters into extracellular medium. Translocation into a host cell requires that effectors associate with specific chaperone proteins within the bacterium. It is proposed that a second translocation–targeting signal ("S2") is formed upon the association of Yops with specific chaperone proteins within the bacterium. Secondary structure within the YopE chaperone-binding (Cb) region requires association with the chaperone SycE (chapter two). The YopE Cb region houses a conserved β–hairpin that contains residues that are important for translocation, but are not required for interaction with SycE (chapter three). This observation supports the hypothesis

that SycE generates a translocation targeting signal (S2) within the YopE Cb region. Here we continue to examine this hypothesis by seeking to identify proteins that recognize the proposed S2 signal.

It is unknown how TTS targeting signals are recognized. The inner membrane injectisome components might act as receptors for these signals, and here we assay the ability of several inner membrane TTS proteins to interact with effectors. Potential insights into effector recognition might be gleaned from understanding of the assembly of the bacterial flagellum. The inner membrane components of injectisomes and flagella are highly conserved, and several proteins are founds to be substrates for export by both systems (Lee and Galan 2004). The *Salmonella* flagellum component FlhA consists of an amino-terminal transmembrane domain which interacts with other membrane-bound export components (McMurry, Van Arnam et al. 2004), and a carboxy-terminal cytoplasmic domain which interacts with soluble export substrates and flagellar chaperones (Minamino and MacNab 2000). It has thus been proposed that FlhA serves as a docking platform for multiple TTS components (McMurry, Van Arnam et al. 2004). The Yersinia TTS protein YscV is related to FlhA (56% amino acid similarity) and may function in a similar manner.

Like YscV, YscU consists of a hydrophobic N-terminus and a cytoplasmic C-terminus. The C-terminal domain undergoes auto-cleavage and responds to injectisome assembly to mediate a switch in the type of substrates secreted. It has been proposed that substrate specificity is switched in response to formation of

the injectisome inner rod (Marlovits, Kubori et al. 2006; Wood, Jin et al. 2008), needle (Journet, Agrain et al. 2003), or both. Needle length is likely governed by YscP, which acts as a molecular ruler to regulate the polymerization the needle subunit YscF (Journet, Agrain et al. 2003; Agrain, Callebaut et al. 2005). A mutation which prevents YscU cleavage (YscUN263A) inhibits secretion of YscP, and needles consequently grow longer (Sorg, Wagner et al. 2007). YscUN263A also abrogated secretion of LcrV, YopB and YopD (Agrain, Callebaut et al. 2005; Sorg, Wagner et al. 2007). The "translocators" LcrV, YopB and YopD are required for effectors to gain entry into host cells (Cornelis and Wolf-Watz 1997; Fields, Nilles et al. 1999; Pettersson, Holmstrom et al. 1999; Marenne, Journet et al. 2003). LcrV localizes at the distal tip of the needle (Mueller, Broz et al. 2005), and interfaces with the pore-forming proteins YopB and YopD, enabling effectors to gain access to the host cell cytoplasm. (Hakansson, Schesser et al. 1996; Cornelis and Wolf-Watz 1997; Francis and Wolf-Watz 1998; Hoiczyk and Blobel 2001; Olsson, Edqvist et al. 2004; Coombes and Finlay 2005; Goure, Broz et al. 2005; Mueller, Broz et al. 2005; Ryndak, Chung et al. 2005; Broz, Mueller et al. 2007). Mutations preventing cleavage of YscU (and the orthologous proteins *Salmonella* SpaS and *E. coli* EscU) likewise halt secretion of LcrV, YopB, YopD and orthologous proteins (Sorg, Wagner et al. 2007; Zarivach, Deng et al. 2008). *Yersinia* containing YscUN263A were capable of exporting the effector YopE nearly as efficiently as wild-type *Yersinia*, and fusion of the YopE N-terminal export signal (S1) to LcrV

restored LcrV secretion (Sorg, Wagner et al. 2007). It was therefore proposed that YscU is critical to recognition of some export substrates. Here I present initial attempts to express the cytoplasmic C-termini of YscU and YscV to determine if these proteins directly associate with YopE.

MATERIALS AND METHODS

Protein expression and purification

A biotinylated version of SycE-YopE, with YopE containing a C-terminal 21-residue biotinylation sequence (KLPAGGGLNDIFEAQKIEWHE), was expressed using pAC-6 in *E. coli* AVB101 cells (Avidity). *In vivo* biotinylated SycE-YopE was purified as previously described (Birtalan, Phillips et al. 2002), except that precipitation was carried out with 50% $(NH_4)_2SO_4$ and the protein was dialyzed in pH 8 Tris buffer.

Yersinia pseudotuberculosis YscVc (containing YscV residues 320-704) and YscUc (containing YscU residues 208-354) were expressed from the vector PH8, which is a modification of pET28b (Novagen) altered to include recombination sites compatible with the Gateway cloning system (Invitrogen). Both proteins contained an amino-terminal histidine tag, thrombin cleavage site, and artefactual residues necessary for recombination (MKHHHHHHHHGGLVPRGSHDQTSLYKKAGF). For YscUc expression bacteria were grown at 37 ºC to mid-log phase, and Luria-Bertani broth (LB), induced with 1 mM IPTG, and grown for 18 hours at 17 ºC, 25 ºC or 37 ºC (see results). Bacteria were harvested by centrifugation and lysed by sonication in lysis buffer (500 mM NaCl, 50 mM sodium phosphate, pH 8.0, 5 mM imidazole, and 10 mM β-mercaptoethanol supplemented with 1 mM PMSF and 1 mg/ml

lysozyme). Lysis buffer additionally supplemented with 1% (v/v) Triton-X 100, 1% (w/v) CHAPS or 0.5% (v/v) Tween-20 was also used to assay the effect of each detergent on YscUc solubility (see results). The lysate was clarified by centrifugation (10 min, 15,000 x g, 4 ºC), and the supernatant was separated from insoluble matter. The insoluble fraction was suspended in 8 M urea for SDS-PAGE analysis. YscVc was expressed as described above, and bacteria grown at 25 ºC and lysed in buffer containing no detergent. Clarified lysate was applied to a Ni^{2+}-chelation column and YscUc was eluted using 333 mM imidazole in lysis buffer.

Binding experiments with biotinylated SycE-YopE and YscVc

Biotinylated SycE-YopE (50 µg) was incubated with 50 µL streptavidin-agarose beads for 3 hours at 4 ºC in 500 µL binding buffer (150 mM NaCl, 50mM sodium phosphate buffer, pH 8.0, 5% glycerol, 10 mM β-mercaptoethanol). Beads were centrifuged (1 minute, 6,000 x g, 4 ºC) and the supernatant, which contained unbound protein, was removed. Purified YscUc (0.2 mg) was diluted 10-fold in binding buffer and applied to SycE-YopE immobilized on streptavidin beads or beads containing no bound protein. Resulting slurries were rocked for 30 min at 4 °C, and washed seven times with 1 ml binding buffer. Bound proteins were removed from beads by boiling in 2x SDS-PAGE sample buffer, and resolved by 12% SDS-PAGE.

Binding experiments with biotinylated SycE-YopE and *Yersinia* lysate

Biotinylated SycE-YopE (125 μg) was incubated with 20 μL streptavidin-agarose beads for 30 minutes at 25 ºC in 500 μL binding buffer (150 mM NaCl, 50mM tris buffer, pH 8.0, 5% glycerol, 1% triton-X 100, 10mM β-mercaptoethanol). Beads were centrifuged (1 minute, 6,000 x g, 4 ºC) and the supernatant, which contained unbound protein, was removed. Beads were washed three times with 700 μL of binding buffer.

Lysates were prepared from *Y. pseudotuberculosis* 126 (*ΔyopE*). This strain had been generated through standard allelic exchange procedures, with the coding region for *yopE* being replaced by the coding region for *kanR* (Birtalan 2004). *Y. pseudotuberculosis* was grown in LB media at 37 ºC until OD_{600}=0.1, and type III secretion was induced by addition of sodium oxalate and $MgCl_2$ to final concentrations of 20 mM each. After 3 hr, bacteria were harvested by centrifugation (10,000 x g, 20 min, 4 ºC) and resuspended in 25 mL binding buffer (supplemented with 1 mM PMSF and 0.5 mM E-64) per liter of bacterial culture, and lysed by sonication. The lysate was clarified by centrifugation (20 min, 30,000 x g, 4 ºC).

Lysates (~500 μL) from wild-type *Y. pseudotuberculosis* cultures (~500 ml) were added to 20 μl streptavidin-agarose beads containing bound SycE-YopE. Resulting slurries were rocked for one hour at 25 °C, washed three times with 700

µl binding buffer. Bound proteins were removed from beads by incubating the beads in 75 µl 8 M urea, 50 mM Tris buffer, pH 8.0 for seven minutes at 25 ºC. Resulting slurries were briefly centrifuged (1 minute, 6,000 x g, 4 ºC) and 65 µl of the eluate was collected and prepared for analysis by mass spectrometry. Analytes were reduced, alkylated, trypsinized, and analyzed by ion trap ms/ms tandem mass spectrometry as described previously (Smolka, Albuquerque et al. 2007).

RESULTS

No proteins identified which bind wild-type YopE, but not YopE-3Ala

Association with SycE promotes the formation of several β-strands and α-helices in the YopE Cb region (chapter two). The first two β-strands form a β-hairpin which is structurally conserved among several chaperone-effector complexes (chapter three). Within this motif, Val23, Glu25, Ser27, Arg29 and Ser32 play a role in YopE translocation, but are not essential for binding to SycE (chapter three). Substantially more wild-type YopE was translocated into HeLa cells than YopE-3Ala (V23A, E25A, and S32A) or YopE-5Ala (V23A, E25A, S2A7, R29A and S32A). This supports the notion that the YopE β1-β2 hairpin constitutes a translocation targeting motif, and I hypothesized that YopE-3Ala and YopE-5Ala were unable to be recognized by a TTS component required for translocation. I sought to discover the receptor for this translocation targeting motif by identifying proteins from *Yersinia* lysates that bind wild-type YopE but neither YopE-3Ala nor YopE-5Ala. I identified several proteins that selectively bound wild-type YopE, but not the mutants, including ribosomal subunits, small molecule transporters, metabolic enzymes and proteases (Fig. 4.1). No TTS components were found to bind wild-type or mutant YopE proteins.

No association detected between YscVc and SycE-YopE

The C-terminus of FlhA (residues 328-692) is sufficient to bind flagellar chaperones and export substrates (Minamino and MacNab 2000) and I sought to determine whether the equivalent YscV domain could bind TTS chaperone-effector complexes. Algorithms that predict protein topology suggest that residues 328-704 of YscV form a cytoplasmic domain (data not shown). This domain, termed "YscVc," was cloned with an amino-terminal histidine tag and expressed in *E. coli* (see materials and methods). YscVc was largely insoluble when bacteria were grown at 37 ºC, and inclusion of 1% (v/v) Triton-X in lysis buffer did not improve solubility. However, bacteria cultured at 25 ºC expressed YscVc predominantly in soluble form (Fig 4.1A). After purification by nickel-affinity chromatography, expression of soluble YscVc was estimated to be 70 mg/L of bacteria. This single purification step yielded protein visible as a single SDS-PAGE resolved band (Fig. 4.1B 'Input').

I sought to determine whether YscVc associated with purified SycE-YopE complexes. Purified YscVc was applied to streptavidin-agarose beads containing SycE-YopE complexes immobilized via site-specific biotinylation of YopE. Very little YscVc was found to associate with immobilized SycE-YopE, and a comparable or greater amount of YscV was found to adhere to streptavidin beads without SycE or YopE (Fig 4.1B). Likewise, histidine-tagged YscVc immobilized on nickel-agarose beads did not bind purified SycE-YopE complexes specifically (data not shown).

The cytoplasmic C-terminal domain of YscU is insoluble when expressed in *E. coli*

The amino-terminus of YscV likely interacts with other inner-membrane components. An association between the flagellar proteins FlhA and FlhB suggests that YscV binds YscU (an ortholog of FlhB). Auto-cleavage in the carboxy-terminal domain of YscU prevents the secretion of the translocator protein LcrV, but fusion of the first 15 YopE residues to LcrV restores secretion (Sorg, Wagner et al. 2007). The length of a flexible linker connecting the cytosolic carboxy-terminal domain to the inner membrane-spanning amino-terminal domain has been shown to be critical for secretion of *E. coli* translocation components (Zarivach, Deng et al. 2008). I sought to express and purify the carboxy-terminal domain of YscU to investigate the mechanism by which it participates in the recognition of TTS export substrates. I expressed residues 208-354 of *Yersinia pseudotuberculosis* YscU ("YscUc") recombinantly in *E. coli*, and found this fragment to be completely insoluble when bacteria were grown overnight at 37 ºC, 25 ºC or 17 ºC. Neither the inclusion of Triton-X 100, CHAPS, nor Tween-20 in lysis buffer improved YscUc solubility (Fig. 4.2) (see materials and methods).

DISCUSSION

In this study I sought to identify proteins that bind to effector translocation targeting signals. I was unable to demonstrate an interaction between YscVc and SycE-YopE complexes. Several explanations are possible to explain the disparity between our results and those reported for FlhA. Primarily, these proteins might play different physiological roles in effector translocation, and YscV might not bind TTS chaperone-effector complexes. It is worth noting that FlhA was found to associate with purified chaperones or export substrates, but associations between FlhA and chaperone-export substrates complexes were not examined (Minamino and MacNab 2000). It is possible that FlhA and YscV only interact with chaperones and export substrates that are not associated with each other, and this possibility should be examined using purified SycE and YopE. Furthermore, a model is emerging wherein FlhA serves as a platform to bind FliJ, a protein which accepts chaperones from the ATPase and recycles the chaperones to bind their cognate export substrates (Fraser, Gonzalez-Pedrajo et al. 2003; Evans, Stafford et al. 2006). It should be determined whether the *Yersinia* FliJ homolog YscO facilitates an interaction between SycE and YscVc.

It is also possible that the truncation used in the YscVc construct did not include residues critical for binding chaperone-effector complexes. Continued investigations of YscV include a construct expressing the intact transmembrane

protein. The expression of intact transmembrane YscV may be enhanced by fusion to the membrane-insertion fusion protein MISTIC (Roosild, Greenwald et al. 2005). A final possibility is that YscVc is misfolded or aggregated. Overnight incubation of YscVc at 4 ºC resulted in precipitation of approximately 50% of soluble YscVc (data not shown). Aggregation of YscVc should be determined by size-exclusion chromatography and static light scattering, and conditions promoting YscVc solubility should be determined.

I was unable to obtain soluble YscUc, and was thus unable to examine the ability of this fragment to bind SycE-YopE complexes. The insolubility of YscUc is surprising considering that similar constructs comprising the carboxy-termini of the *Salmonella* ortholog SpaS and *E. coli* EscU were soluble (Zarivach, Deng et al. 2008). These three proteins were expressed from pET28-related plasmids, and bacteria were lysed in similar buffers (see materials and methods). Soluble SpaS and EscU fragments were obtained from bacteria lysed by a French press, in contrast to the sonication procedure used for YscUc. Lysis procedure should be examined as a possible means of improving YscUc solubility. Furthermore, continued studies of YscU include expression of intact transmembrane YscU, which may again be aided by fusion with MISTIC (Roosild, Greenwald et al. 2005).

In addition to YscV and YscU, other injectisome components must not be overlooked as potential receptors for effector targeting signals. Among the potential receptors is the inner-membrane protein YscR, which is predicted to

contain four transmembrane domains and a single 84-residue cytosolic domain (Fields, Plano et al. 1994). Little is known about the function of YscR, although the protein has been shown to be essential for the secretion of several virulence proteins (Fields, Plano et al. 1994). Data describing the *E. coli* YscR homolog EscR suggest that YscR associates with itself, YscU, and the inner-membrane protein YscS (Creasey, Delahay et al. 2003; Riordan and Schneewind 2008).

Flagella and TTS injectisomes harbor ATPases at the inner membrane, and while ATP hydrolysis enhances and translocation of Yops (Iino 1969; Emerson, Tokuyasu et al. 1970; Eichelberg, Ginocchio et al. 1994; Woestyn, Allaoui et al. 1994; Tamano, Aizawa et al. 2000), ATP hydrolysis is not absolutely required for translocation in the flagellar system (Minamino and Namba 2008; Paul, Erhardt et al. 2008). Several TTS ATPases have been reported to bind and unfold export substrates (Thomas, Stafford et al. 2004; Akeda and Galan 2005), but I was unable to demonstrate an interaction between the *Yersinia* ATPase YscN and SycE-YopE complexes *in vitro* (chapter two). It is possible that additional components are necessary to mediate an interaction between YscN and chaperone-effector complexes. One candidate for this task is YscQ, a 34 kD protein predicted to lack transmembrane domains. YscQ has been shown to interact with YscU, YscN and the YscN regulator YscL (Jackson and Plano 2000). The association of YscQ with YscU, as well as the ATPase export machinery positions YscQ as a possible receptor for effector targeting signals. It should be determined whether YscQ associates with SycE-YopE complexes.

Sufficient experimental evidence exists to predict a set of proteins that might recognize effector translocation targeting signals. In this study I describe efforts to predict such candidates, and I present preliminary binding experiments. Association assays that utilize purified components face technical difficulties in obtaining a sufficient quantity of soluble proteins. Expression of recombinant injectisome components has proven difficult, and solubility of many TTS proteins has relied on truncations, refolding, and fusion to solubility-enhancing partners. Association assays that do not require purification of injectisome components may overcome substantial hurdles, and additionally remove the bias inherent when purified components are used. I previously utilized immobilized SycE-YopE complexes and *Yersinia pseudotuberculosis* lysate to identify proteins that associate with the chaperone-effector complex. Most prominent among these was DnaK (M. Fakhouri and P.G., unpublished results), a heat shock protein essential for type III secretion (Takaya, Tomoyasu et al. 2004). DnaK assists with protein refolding during the heat shock response, and is known to nonspecifically interact with unfolded and misfolded proteins (Hightower 1990; Hartl 1996; Slepenkov and Witt 2002). Intriguingly, protein secretion by the *Salmonella enterica* serovar Typhimurium flagellar and TTS systems was shown to require DnaK (Takaya, Tomoyasu et al. 2004). Purified DnaK was found to associate with free YopE and SycE-YopE complexes, but no DnaK-SycE association was detected (A. Raposo and P. G., unpublished results). A YopE fragment lacking translocation targeting motifs (YopE 81-219) was found to bind DnaK nearly as well as a SycE-YopE

fragment (SycE-YopE1-80) that contains the translocation targeting signals (A. Raposo and P. G., unpublished results). The DnaK-YopE interaction is therefore unlikely to involve the specific recognition of effector targeting signals, and may simply demonstrate the propensity of DnaK to bind exposed hydrophobic residues. It is unknown how DnaK contributes to protein export by the TTS system, and it remains possible that TTS components require the DnaK for proper folding prior to injectisome assembly.

Our investigations, particularly in chapters two and three, have advanced our understanding of effector targeting signals. The receptor for these signals remains unidentified, and may involve a complex of multiple injectisome components. The possibility of a multi-partite recognition complex, along with the poor solubility of many components likely complicates attempts to reconstruct the effector-recognition complex *in vitro*. Current investigating the TTS components highlights in this chapter have had some success solublizing the proteins through refolding procedures, truncations, or fusions to solubility-enhancing proteins (P.G. et al., unpublished results). In addition to strategies that target specific proteins, continued effort should be placed in techniques that are unbiased in seeking chaperone-effector binding partners, such as "pull-down" assays from *Yersinia* lysate, far-Western blotting, and yeast (or bacterial) two-hybrid assays. Each method presents unique technical challenges. However, the availability of YopE mutants that are not translocated (namely YopE-3Ala and YopE-5Ala) provides useful negative controls to allow one to identify interactions

that specifically recognize the chaperone-mediated effector targeting signal.

FIGURES AND TABLES

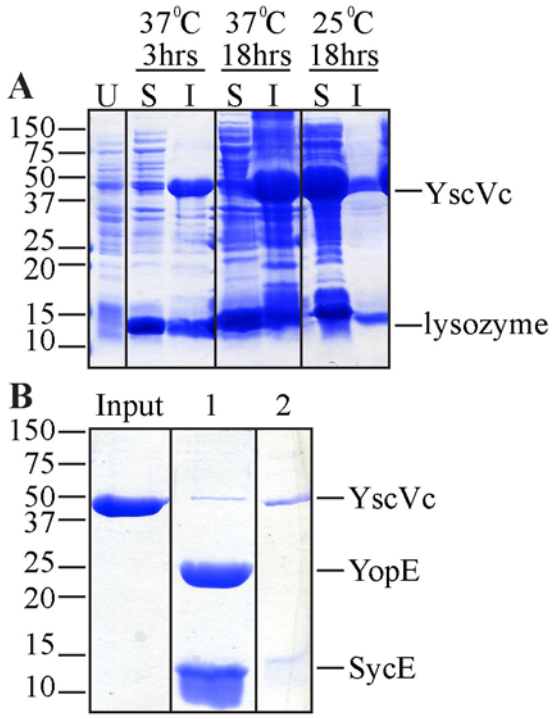

Figure 4.1. YscVc is soluble when expressed at 25 ºC and does not associate with SycE-YopE.
A. SDS-PAGE demonstrating YscVc (46 kD) expression and solubility. Bacteria were grown at 37 ºC or 25 ºC for three hours or 18 hours. Lysate from uninduced bacteria ('U'), soluble ('S') and insoluble ('I') fractions are shown. Lysozyme (14 kD) was used to facilitate bacterial lysis.
B. **Lack of association between SycE-YopE and YscVc.**
Purified YscVc ('Input') was applied to biotinylated SycE-YopE (15 kD and 26 kD, respectively) complexes immobilized on streptavidin beads ('1') or streptavidin beads lacking attached SycE-YopE ('2'). Beads were washed to remove unbound proteins (see materials and methods). SycE, YopE, and bound proteins were removed from the beads by boiling in SDS-PAGE sample buffer.

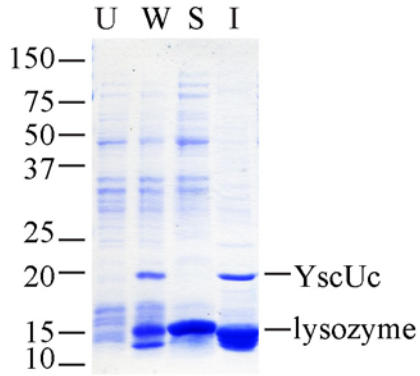

Figure 4.2. YscUc Insolubility.
SDS-PAGE demonstrating YscUc (21 kD) expression and solubility. Bacteria were grown at 25 ºC for 18 hours. Lysate from uninduced bacteria ('U'), induced whole cell lysates ('W'), soluble ('S') and insoluble ('I') fractions are shown. Lysozyme (14 kD) was used to facilitate bacterial lysis.

Table 4.1. Proteins that associate with SycE-YopE as identified by mass spectrometry.
Yersinia proteins that associate with wild-type YopE, but not YopE-3Ala or YopE-5Ala. Proteins were identified by mass spectrometry from *Yersinia pseudotuberculosis* lysate incubated with wild-type YopE, YopE-3Ala or YopE-5Ala immobilized on streptavidin beads. Proteins that bound streptavidin beads lacking bound YopE were excluded from this list.

Accession Number	Name	Function
YP_068883.1	HmuU	ABC hemin transporter
YP_068891.1	None	putative methylenetetrahydrofolate reductase
YP_068925.1	ExrB	single-strand DNA-binding protein
YP_068948.1	GroEL	chaperonin
YP_068984.1	RplI	50S ribosomal protein L9
YP_069000.1	none	UDP-N-acetylmuramate: L-Ala-D-Glu-meso-diaminopime late ligase
YP_069254.1	AceE	pyruvate dehydrogenase subunit E1
YP_069506.1	YbaW	Putative 4-hydroxybenzoyl-CoA thioesterase
YP_069525.1	AcrA	acridine efflux pump, membrane fusion
YP_069902.1	none	putative iron ABC type transport, ATP-binding protein
YP_069984.1	OmpA	porin, diffusion of nonspecific small solutes across the outer membrane
YP_070246.1	YecC	ATP-binding protein, putative amino-acid ABC transporter
YP_070890.1	HtpX	Zn-dependent protease with chaperone function
YP_072111.1	ClpB2	Proteinase, ATPase with chaperone activity, ATP-binding subunit
YP_072169.1	RplN	50S Ribosomal protein L14
YP_072186.1	RpsL	30S ribosomal protein S12

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V.

Appendix: Remaining Experiments

SUMMARY

The ability of pathogenic *Yersinia* spp. to establish and maintain infections is contingent on the bacterium's capacity to modulate targets within host cells. These microbes possess virulence proteins, termed Yops" for "*Yersinia* Outer Proteins", which disable critical host defenses. Yops exit the bacterium through the needle-like injectisome complex, which most likely delivers virulence proteins directly into the cytosol of targeted cells (Ghosh 2004). This type III secretion (TTS) system consists of over 20 injectisome components, six Yops which gain entry into host cells, and chaperone proteins which are not translocated and remain in the bacterial cytosol. Individual TTS chaperones bind one or a few specific Yops within the bacterium. The translocation of at least four of the six translocated Yops requires their prior association with specific chaperones within the bacterium. We examined the *Yersinia pseudotuberculosis* SycE-YopE chaperone-effector complex to ascertain the mechanism by which this association promotes effector translocation, and we proposed that chaperones target Yops to a TTS component required for translocation. Using NMR spectroscopy, we compared the structure and dynamics of YopE in its free and chaperone-associated states. We found that SycE promotes a localized disorder-to-order transition within the chaperone-binding (Cb) region of YopE (residues 15-80) (chapter two). The Cb region lacks detectable secondary structure in the absence of SycE, and upon binding SycE the Cb region forms several short α-helices and

β-strands. The first two β-strands form a hairpin which contains a set of stereochemically conserved side-chains. We generated two YopE mutants which convert sets of residues in this β-strand hairpin to alanine: YopE-3Ala (V23A, E25A and S32A) and YopE-5Ala (V23A, E25A, S27A, R29A and S32A). The translocation of either variant into HeLa cells was significantly impaired (chapter three). Neither set of mutations prevented YopE from associating with SycE, and the disruption of two hydrogen bonds in the YopE-5Ala construct (S72 and R29) only slightly reduced the chaperone-effector affinity. The observations that mutations in V23, E25, S27, R29 and S32 disrupt YopE translocation but not its association with SycE supports the hypothesis that these residues target the effector to another TTS component. We are seeking to identify proteins which recognize these YopE residues (chapter four).

Our conclusion that mutations in V23, E25, S27, R29 and S32 disrupt translocation is based upon experiments wherein YopE (wild-type, 3Ala or 5Ala) is translocated from *Yersinia* into HeLa cells. Briefly, HeLa cells are selectively lysed by incubation in a buffer containing 1% (v/v) Triton-X 100, and translocated YopE is quantified from anti-YopE Western blots. We sought to determine whether mutation of V23, E25, S27, R29 and S32 altered the stability of YopE within HeLa cells. Within mammalian cells, YopE translocated from some *Yersinia* strains has a half-life that is largely driven by ubiquitin-mediated degradation (Ruckdeschel, Pfaffinger et al. 2006). If YopE-3Ala or YopE-5Ala

are degraded more quickly than wild-type YopE, it would falsely appear that the mutated versions were translocated less efficiently than wild-type. The YopE polyubiquitin-receptor sites have been mapped to Lys62 and Lys75 found in the C-terminal half of the Cb region (Ruckdeschel, Pfaffinger et al. 2006; Hentschke, Trulzsch et al. 2007). Strains with residues other than lysine in these positions are not susceptible to ubiquitin-mediated degradation (Hentschke, Trulzsch et al. 2007). Mutations in YopE-3Ala and YopE-5Ala did not disrupt residues in position 62 or 75, and are therefore unlikely to alter ubiquitin-mediated YopE degradation. Furthermore, YopE variants used for our translocation experiments were derived from *Y. pseudotuberculosis* strain 128 and contain arginine and glutamine at positions 62 and 75, respectively. We found that the level of YopE translocated into HeLa cells was not affected by the proteosome inhibitor MG-132 (data not shown), and it is therefore unlikely that any YopE variant used in our studies undergoes significant ubiquitin-mediated degradation in HeLa cells.

Although it is highly unlikely that the proteosome degrades YopE-3Ala and YopE-5Ala more quickly then wild-type YopE, it is possible that the mutated versions are less stable in HeLa lysates. We previously demonstrated that the 100 N-terminal YopE residues, including those mutated in YopE-3Ala and YopE-5Ala, are disordered in the absence of SycE. It is therefore unlikely that mutations that convert V23, E25, S27, R29 and S32 to alanine alter the structure of translocated YopE.

Although YopE-3Ala and YopE-5Ala are unlikely to be structurally destabilized or degraded more quickly than wild-type YopE, we cannot rule out the possibility that an unidentified phenomenon occurring subsequent to translocation is affecting the amount of mutated YopE detected in Western blots. Such a phenomenon would artificially appear to report a variation in the quantity of translocated YopE.

For this reason we sought to establish that substitution of V23, E25, S27, R29 and S32 to alanine does not affect the amount of YopE detected from HeLa lysates, as assayed by Western blotting. We therefore attempted to use syringe loading to introduce equal quantities of YopE, YopE-3Ala and YopE-5Ala into HeLa cells. This technique introduces purified proteins into mammalian cells by disrupting cell membranes by the generation of shear forces (Clarke and McNeil 1992). Cells and protein to be loaded are mixed and repeatedly aspirated and expelled through a narrow needle. We syringe loaded YopE into HeLa cells in order to compare the stability of the wild-type protein to YopE-3Ala and YopE-5Ala. As a negative control, cells were incubated in the presence of wild-type YopE, but were not subjected to the syringe loading procedure. Western blots of these samples reveal a comparable amount of YopE from cells regardless of whether they were syringe loaded (Fig. 5.1). It is unlikely that purified YopE is internalized into HeLa cells without the membrane destabilization provided by the syringe loading procedure. Rather, we speculate that extracellular YopE is adhering membranes of HeLa cells. The hypothesis that extracellular YopE

adheres to cell membranes is supported by the observation that a small amount of YopE secreted from Δ*yopB Yersinia* in the presence of HeLa cells is present in HeLa lysates (Fig. 3.6). The Δ*yopB Yersinia* strain is unable to form pores in targeted cells, and it is highly unlikely that YopE secreted from these bacteria is internalized into HeLa cells (Nordfelth and Wolf-Watz 2001). It should be noted that papain did not reduce the amount of YopE detected from syringe loaded HeLa cells or HeLa cells incubated with YopE that did not undergo the syringe loading procedure, even though enough papain was added to completely digest YopE added to the cells (data not shown). This is consistent with our observation that YopE secreted from Δ*yopB Yersinia* is not reduced by treatment with papain or proteinase K (chapter three), but conflicts with previously published observations (Nordfelth and Wolf-Watz 2001). It is possible that extracellular YopE adheres to HeLa cell membranes in a manner which protects the protein from digestion, but it is unclear why our results are inconsistent with those previously published. Alternative procedures are available for the introduction of YopE into HeLa cells, such as scrape loading (McNeil, Murphy et al. 1984), scratch loading (Swanson and McNeil 1987), and bead loading (McNeil and Warder 1987). However, the usefulness of these techniques may be limited when applied to proteins that adhere to cell membranes.

We also attempted to introduce YopE into HeLa cells by transfection. YopE was expressed from pcDNA3.1+, and as a control for transfection efficiency, this plasmid encoding YopE was co-transfected with p(κβ)3-INF β-

Luc, a plasmid which constitutively expresses firefly luciferase. YopE was not detected from HeLa cells when lysates were examined by Western blot (data not shown). We measured the luminescence of the samples to determine if the transfection was successful. Luminescence was detected from cells transfected with p(κβ)3-INF β-Luc and pcDNA3.1+ lacking YopE as soon as 11 hours after transfection (Fig. 5.2A), indicating that the plasmids were successfully introduced into the HeLa cells. However, luminescence was not detected at any time point from cells transfected with pcDNA3.1+ containing YopE (Fig. 5.2A). YopE is cytotoxic to HeLa cells, and we speculated that YopE-mediated cell death might be the cause of the abrogated luminescence. We therefore used trypan blue exclusion to measure the number of viable cells after transfection. We found that the number of living cells transfected with YopE decreased after transfection, while the number of living cells transfected with pcDNA3.1+ lacking YopE increased (Fig. 5.2B).

In order to overcome the cytotoxic properties of YopE, we generated a mutation (R144A) which disables YopE's ability to enhance the rate of GTP hydrolysis by Rho protein (Von Pawel-Rammingen, Telepnev et al. 2000). Mutation of the arginine finger (Arg 144) within YopE's Rho-GAP (Rho-GTPase activating) domain eliminates *in vitro* YopE GAP activity and abolishes the YopE cytotoxic effect (Black and Bliska 2000). Surprisingly, we found that the R144A mutation was unable to restore luciferase expression (Fig. 5.2A). The viability of

cells transfected with YopER144A was not determined, and should be examined to verify that the YopE cytotoxic effect has been eliminated. Alternative reporters, such as green fluorescent protein, should also be examined to find a suitable means of determining transfection efficiency. Lastly, transfected HeLa cells should be examined by an anti-YopE western blot to determine if YopER144A is expressed in transfected HeLa cells.

MATERIALS AND METHODS

YopE expression and purification

Wild-type YopE, YopE-3Ala and YopE-5Ala were co-expressed with SycE from pET28b, yielding YopE fused to a C-terminal his tag (LEHHHHHH). Bacteria were grown at 37 ºC to mid-log phase in Luria broth, induced with 1 mM IPTG, and grown for 18 hours at 25 ºC and harvested by centrifugation (10,000 x g, 20 min, 4 ºC). Bacteria were then lysed by sonication in lysis buffer (500 mM NaCl, 50 mM sodium phosphate, pH 8.0, 5 mM imidazole, 5 mM $MgCl_2$, and 10 mM β-mercaptoethanol supplemented with 1 mM PMSF and 20 μg/ml DNAse). The lysate was clarified by centrifugation (20 min, 30,000 x g, 4 ºC), and SycE-YopE was applied to a Ni^{2+}-chelation column and eluted using a 5-500 mM imidazole gradient in lysis buffer. The eluate was concentrated by ultrafiltration (MWCO 30 kD), and further purified by size-exclusion chromatography (Superdex 75) in 500 mM NaCl, 50 mM sodium phosphate, pH 8.0, 10 mM β-mercaptoethanol. SycE-YopE was denatured in 8 M urea, 50 mM sodium phosphate, pH 8.0, and bound to Ni^{2+}-NTA agarose beads. Denatured YopE was eluted from these beads with 8 M urea, 50 mM sodium phosphate, pH 4.0, and renatured by dilution in 1 M arginine, 500 mM NaCl, 50 mM Tris, pH 8.0, 5 mM β-mercaptoethanol, 5 mM EDTA at a final concentration of 1 μM (determined by A_{280}). Renaturation solutions were stirred vigorously for a minimum of 1 hr at 4

ºC, and then dialyzed in 500 mM NaCl, 50 mM Tris pH 8.0, 5 mM β-mercaptoethanol, and 5 mM EDTA. Renatured and aggregated proteins were separated by size-exclusion chromatography (Superdex 200) in 50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol, and concentrated by ultrafiltration (MWCO 10 kD).

Syringe loading of YopE into HeLa cells

HeLa cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum (FBS), glutamine, streptomycin, and penicillin in a 5% CO_2 humidified incubator at 37 ºC. HeLa cells were trypsinized and resuspended in DMEM at a concentration of 2.2 X 10^6 cells per ml DMEM. Purified YopE (260 µl, 0.23 µg/µl) was added to 900 µl HeLa cells, and 110 µl 20% (w/v) Pluronic F68 was added to the suspension. Syringe-loaded samples were slowly aspirated and expelled (~ 1 min per aspiration and expulsion cycle) six times through a 30.5 gauge syringe needle. Control samples were not drawn through a syringe, but were incubated for six minutes at 25 ºC. Cells were centrifuged for three minutes (1,000 x g, 25 ºC) and the supernatant was removed by aspiration. Cells were washed three times with 500 µl PBS (25 ºC), and following the aspiration of the last wash, papain was added to digest extracellular proteins (100 µl from a 0.25 mg/ml papain stock in PBS with 2.0 mM DTT). This digestion proceeded for 20 minutes at room temperature, and was stopped by the addition of E-64 (90 µl

from a 10 µg/ml stock). Cells were incubated in E-64 for five minutes before centrifugation (five minutes, 1,000 x g, 25 ºC). Supernatants were aspirated and cells were incubated in 50 µl lysis buffer (1% Triton-X 100, 10% glycerol, 150 mM NaCl, 10 mM Tris pH 8.0), for five minutes at 25 ºC. Cell lysates were clarified by centrifugation (10 minutes, 16,000 x g, 4 ºC). Total protein content was quantified by a Lowry assay (Bio-RAD DC-protein detection kit) using a 96-well plate reader. Lysate volumes were adjusted with lysis buffer to make protein concentrations equivalent among samples. SDS-page sample buffer (4X) was added to the samples, which were then boiled for 10 minutes. Samples were centrifuged for 1 minute at 16,000 x g (room temperature), and were analyzed by Western blotting as described previously (chapter three).

Transfection of YopE into HeLa cells

The coding sequence of YopE was inserted into pcDNA3.1+, and the point mutation R144A was introduced using the Quickchange method (Stratagene). Six-well plates of 80-90% confluent HeLa cells were washed twice with 37 ºC PBS and cell monolayers were overlaid with 1.9 ml DMEM (37 ºC). Two µg pcDNA3.1+ containing YopE or YopE-R144A was combined with 1 µg p(κβ)3-INF β-Luc, (which constitutively expresses firefly luciferase) and 5 µl Fugene HD (Roche), and DMEM was added to raise the total volume to 100 µl. For a negative control, 2 µg pcDNA3.1+ lacking YopE was used in the place of

pcDNA3.1+ containing YopE or YopE-R144A. Transfection mixtures were mixed by aspirating and expelling with a pipette five times, and the mixtures were added to HeLa monloayers. Cells were incubated in a 5% CO_2 humidified incubator at 37 ºC for 4.5, 7, 11 or 23 hours. When harvested, cells were washed once with 1 ml PBS (4 ºC), and overlaid with 250 µl lysis buffer (1% Triton-X 100, 10% glycerol, 150 mM NaCl, 10 mM Tris pH 8.0). Cells were scraped to facilitate removal from the plate, and lysates were clarified by centrifugation (10 minutes, 16,000 x g, 4 ºC). Luminescence was measured using a firefly luciferase assay kit (Promega) according to the manufacturer's instructions.

FIGURES

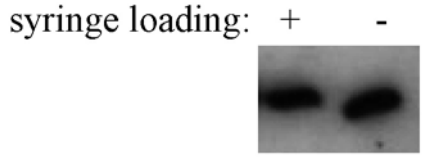

Figure 5.1. YopE adheres to HeLa cells without syringe loading.
YopE measured by anti-YopE Western blot from HeLa cell lysates. HeLa cells were syringe loaded in the presence of YopE ("+"), or incubated in DMEM containing YopE without syringe loading ("-"). Samples were within a linear range of measurement.

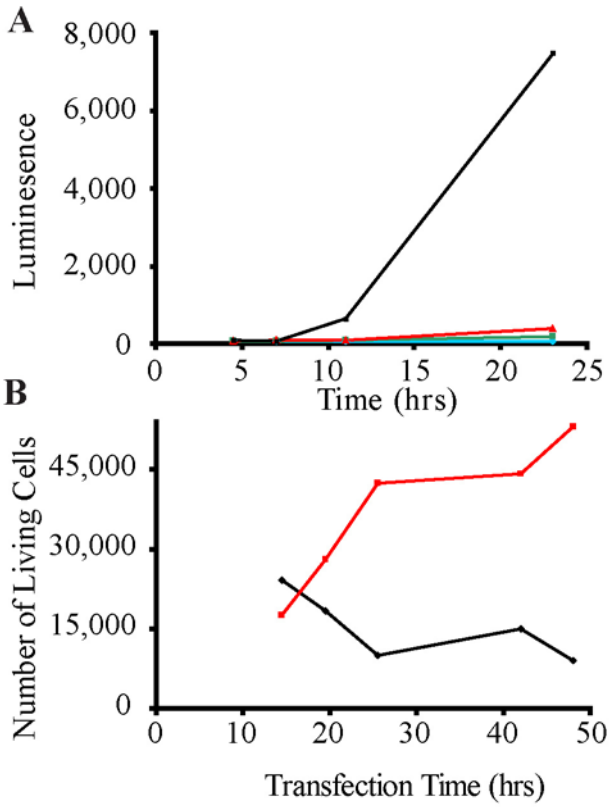

Figure 5.2. Transfection of YopE inhibits expression of co-transfected luciferase.

A. Luminescence measured from lysates of HeLa cells transfected with luciferase + control plasmid (black), luciferase + YopE (green), or luciferase + YopE-R144A (red). Luciferase substrate without cell lysate (cyan) is measured as an indicator of the level of background luminescence. Luminescence is measured in arbitrary units.

B. Viability of HeLa cells transfected with YopE (black) or control plasmid (red). Viability is measured by counting the number of living cells as assayed by trypan blue staining.

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