THE VIRULENCE CHAPERONE NETWORK OF *SALMONELLA ENTERICA*

THE VIRULENCE CHAPERONE NETWORK ASSOCIATED WITH THE SPI-2 ENCODED TYPE THREE SECRETION SYSTEM OF *SALMONELLA ENTERICA*

By

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TITLE: The virulence chaperone network associated with the SPI-2 encoded type three secretion system of *Salmonella enterica*

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Abstract

Bacteria employ virulence mechanisms to promote fitness that are generally detrimental to a host organism. The Gram-negative pathogen *Salmonella enterica* utilizes type three secretion systems (T3SS) to inject proteins termed effectors into the host cell cytoplasm where normal cellular function is modified. The coordinated T3SS assembly, and delivery of effectors to the cytoplasmic face of the T3SS is aided by virulence chaperones. The interaction of effector-chaperone complex with the T3SS occurs via an ATPase protein, where the complex is dissociated and the effector is unfolded, presumably for passage through the T3SS. The virulence chaperone network associated with the *Salmonella* pathogenicity island two (SPI-2) encoded T3SS has not been fully characterized. Additionally, the T3SS ATPase protein encoded within SPI-2, SsaN, has yet to be examined for functional motifs or a precise role in effector secretion. The contents of this thesis describe the characterization of two novel virulence chaperones, SrcA and SscA, and the T3SS ATPase SsaN. SrcA is a virulence chaperone for the effector substrates SseL and PipB2, and adopts the characteristic horseshoe-like structure common amongst effector chaperones. SscA is a chaperone for the translocon component SseC of the T3SS structure, and both proteins impact the regulation of SPI-2 promoters. The structure of SsaN resembles other T3SS ATPases, although different conformations exist between the structures, potentially highlighting regions with T3SS function. Additionally, an N-terminal domain was found to be dispensable for membrane localization, and residues within the predicted hexamer model impact effector secretion. These results identify novel virulence chaperones essential for T3SS function, and characterize the T3SS ATPase protein encoded within SPI-2. These findings greatly expand our knowledge of the virulence mechanisms utilized by *S. enterica*.

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Pathogenicity island (PAI)

***Salmonella* pathogenicity island (SPI)**

***Salmonella* pathogenicity island one (SPI-1)**

***Salmonella* pathogenicity island two (SPI-2)**

Type three secretion system (T3SS)

Gastrointestinal (GI)

Non-typhoidal *Salmonella* (NTS)

***Salmonella enterica* serovar Typhi (*S.* Typhi)**

***Salmonella enterica* serovar Typhimurium (*S.* Typhimurium)**

Definitive phage type 104 (DT104)

***Salmonella* genomic island one (SGI-1)**

Acid shock proteins (ASP)

Microfold cells (M cells)

***Salmonella* containing vacuole (SCV)**

Pathogen-associated molecular patterns (PAMPs)

Toll-like receptor (TLR)

Guanine nucleotide exchange factor (GEF)

Open reading frame (ORF)

Inner membrane (IM)

Outer membrane (OM)

Angstrom (Å)

Cytoplasmic ring (c-ring)

Green fluorescent protein (GFP)

Enteropathogenic *Escherichia coli* (EPEC)

Enterohemorrhagic *Escherichia coli* (EHEC)

Locus of enterocyte effacement (LEE)

Adenosine triphosphate (ATP)

Guanosine triphosphate (GTP)

GTPase activating protein (GAP)

Valosin-containing protein (VCP)

Skp, Cullin, F-box (SCF)

KiloDalton (kDa)

Isoelectric point (pI)

Chaperone binding domain (CBD)

Mass spectrometry (MS)

Liquid chromatography (LC)

Stable isotope labelling with amino acids in cell culture (SILAC)

Low phosphate and magnesium (LPM)

Terrific broth (TB)

Luria broth (LB)

Competitive index (CI)

M2-Agarose beads conjugated with anti-FLAG antibody (F-gel)

Ethylenediaminetetraacetic acid (EDTA)

Phosphate buffered saline (PBS)

Protease inhibitors (PI)

Hemagglutinin (HA)

Horseradish peroxidase (HRP)

Intergenic region (IGR)

Sodium dodecyl sulfate (SDS)

Polyacrylamide gel electrophoresis (PAGE)

Bovine serum albumin (BSA)

Colony forming unit (CFU)

Multiplicity of infection (MOI)

Wild type (Wt)

Host cell membrane (HCM)

Tris buffered saline with Tween-20 (TBST)

Chapter I – Introduction

Chapter I – Introduction

Salmonella

The genus *Salmonella* is comprised of Gram-negative, facultative, rod-shaped bacteria with isolates found worldwide in a multitude of environments and hosts. The two predominant species of *Salmonella* are the commonly pathogenic *Salmonella enterica*, and the mostly commensal *Salmonella bongori*. Though only two main species of *Salmonella* have been identified, they branch into thousands of serovars with diverse genomic islands, antibiotic resistances, secretion systems, and host specificities (Gulig & Doyle, 1993, Kingsley & Baumler, 2002, Hall, 2011, Haraga et al., 2008). The divergence of *Salmonella* from fellow Enterobacteriaceae *Escherichia coli* is believed to have occurred mainly through the acquisition of pathogenicity islands (PAI). Of these PAIs, the most notable contributors to virulence are *Salmonella* pathogenicity island-1 and 2 (SPI-1 and SPI-2), which encode type three secretion systems (T3SS) capable of secreting proteins termed effectors into host cells.

The majority of *Salmonella enterica* serovars cause gastroenteritis via infection of the gastrointestinal (GI) tract, resulting in approximately 1.4 million infections, 168 000 hospital visitations, and 580 deaths costing a total of $3 billion annually in the United States alone (WHO 2005, fact sheet 139). These infections are a result of non-typhoidal *Salmonella* (NTS) infections, whereas typhoidal infections are caused by serovars Typhi (*S.* Typhi) and Paratyphi and result in a longer, more systemic infection such as typhoid fever. *S.* Typhi infects approximately 22 million people per year resulting in approximately 216 000 deaths, predominantly in school-aged children. These infections typically occur in developing nations lacking hygiene practices capable of preventing transmission of pathogens through the fecal-oral route (Crump & Mintz, 2011, Crump et al., 2004). The treatment of *S. enterica* infection is

becoming increasingly difficult as a result of antibiotic resistant strains such as *S. enterica* serovar Typhimurium (*S.* Typhimurium) definitive phage type 104 (DT104) which contains the highly mobile, antibiotic resistance encoding, *Salmonella* genomic island one (SGI-1) (Quinn et al., 2006). Increasing prevalence and severity of *Salmonella*-mediated infections necessitates continued investigation into its virulence mechanisms, with aims to develop novel treatment strategies.

Salmonella pathogenesis and treatment

The initiation of *S. enterica* infection occurs shortly after ingestion of contaminated food or water. *S. enterica* is then exposed to the highly acidic environment of the stomach, where an acid response system is activated composed of acid shock proteins (ASP), which aid in the prevention or repair of acid damaged proteins, and are encoded within the *atr* and *fur* operons (Garcia-del Portillo et al., 1993). After passage to the small intestine, invasion of intestinal epithelial cells proceeds by *Salmonella*-mediated endocytosis (Francis et al., 1992, Giannella et al., 1973a, Giannella et al., 1973b). This occurs mainly due to the function of the SPI-1 encoded T3SS, where secreted bacterial effectors hijack host cytoskeletal function (Fu & Galan, 1998, Hayward & Koronakis, 1999, Galan & Zhou, 2000). Additionally, *S. enterica* is pinocytosed by microfold cells (M cells) which are scattered along the intestinal epithelium and directly sample intestinal contents (Jones et al., 1994, von Rosen et al., 1981). Once in the lamina propria, *S. enterica* is able to disrupt tight junctions which likely contributes to increased uptake (Jepson et al., 1995, Takeuchi, 1967). *Salmonella* is also actively phagocytosed by macrophages where the process of phagosome-lysosome fusion begins, but is avoided due to effector functions preventing fusion and acting to enhance intracellular survival (Furness & Ferreira, 1959, Haraga

et al., 2008). The presence of *S. enterica* in the host cell is restricted to a phagosome-like compartment known as the *Salmonella* containing vacuole (SCV) (Finlay & Falkow, 1988). Once *Salmonella* has been internalized, the SPI-2 encoded T3SS and its effector arsenal are largely responsible for survival and maintenance of the SCV and for preventing phagosome-lysosome fusion within the host cell, which would normally result in bacteria lysis (Coombes et al., 2007, Shea et al., 1996, Waterman & Holden, 2003). At this point *S.* Typhi and Paratyphi are able to cause a systemic infection due to their ability to reduce host cell recognition (Calva & Ruiz-Palacios, 1986, Julia et al., 2000). While incidences of systemic NTS infection can occur, the majority of NTS infections are localized to the intestinal lumen and lamina propria (Sirinavin et al., 2001, McCormick et al., 1995). A host inflammatory response to *S. enterica* is generated through recognition of pathogen-associated molecular patterns (PAMPs) and shedding of the intestinal mucosa occurs resulting in nausea, cramps, and diarrhea. PAMPs from bacteria such as *Salmonella* are recognized by toll-like receptors (TLRs), such as TLR-5 in the case of flagellin recognition (Hayashi et al., 2001). TLR signalling is a complex process that leads to the transcription of inflammatory response genes, often through the host transcriptional regulator NF-κB (Akira & Takeda, 2004). NF-κB activation is commonly inhibited by bacterial effectors as a means to subvert the immune response (Le Negrate et al., 2008, Gao et al., 2009, Pearson et al., 2011). Despite our extensive understanding of the process and prognosis of *Salmonella* pathogenesis, treatment strategies have largely remained stagnant.

Since the 1940s, antibiotics have been used in the treatment of bacterial infections, however bacterial resistance has emerged at equal pace with the development of new antibiotic treatment regimes. Chloramphenicol was one of the first documented antibiotic treatments for *Salmonella* infections although resistance quickly developed in *S.* Typhi (Agarwal, 1962). One

of the most resistant strains of *Salmonella* is *S.* Typhimurium DT104 which harbours the SGI-1 island encoding resistance genes for sulfonamide, ampicillin, chloramphenicol, tetracycline, florfenicol, streptomycin, and spectinomycin (Carattoli et al., 2002, Lawson et al., 2002). SGI-1 is a 27.4kb mobile genetic insertion element that is the most common source of antibiotic resistance in *S. enterica*, and exists in 11 different variations (SGI-1A,B,C,D,F,G,I,M,N,O, and R) each coding for a unique set of genes for antibiotic resistance (Hall, 2011). The majority of NTS infections can be successfully treated with fluids and rest, however persistent infections and typhoid fever should be treated with antibiotics upon positive identification (de la Cabada Bauche & Dupont, 2011). Current antibiotic treatments for persistent *Salmonella* infections include ciprofloxacin, rifamixin, or azithromycin, although pathogen resistance to these relatively new antibiotics has already been detected (de la Cabada Bauche & Dupont, 2011, Harish & Menezes, 2011, Hall & MacLean, 2011, Sun et al., 2011). In order to develop new therapeutics for *Salmonella* infection treatment, extensive research is focused on their virulence mechanisms that are often encoded in PAI.

Salmonella pathogenicity islands

The acquisition of PAI and other genomic elements through horizontal transfer is a major evolutionary advantage in bacterial species. Many virulence-associated proteins in *Salmonella* are encoded on PAIs known as SPIs. The most well characterized SPIs are SPI-1 and SPI-2 which each encode a T3SS to facilitate invasion and subsequent intracellular survival in eukaryotic host cells (Groisman & Ochman, 1993, Mills et al., 1995, Akbar et al., 2003, Shea et al., 1996, Hensel et al., 1998). Nineteen additional SPIs have been characterized to date

encoding various virulence-associated genes, with their distribution spread throughout the *Salmonella* serovars (Hensel, 2004).

The genetic content of SPI-3 varies throughout the *Salmonella* serovars, with the majority containing a magnesium uptake operon, *mgtCB*, required for growth in low magnesium media (Blanc-Potard & Groisman, 1997). SPI-4 has recently shown to be important for virulence and is co-regulated with SPI-1. It contains the *siiABCDEF* operon which encodes a secretion system for export of SiiE, a large fimbrial-like protein suggested to interact with the host cell surface prior to invasion (Gerlach et al., 2008). SPI-5 contains the secreted effectors *pipB* and *sopB*, which are secreted through the SPI-2 and SPI-1 secretion systems respectively (Gerlach et al., 2008, Galyov et al., 1997, Darwin et al., 2001). SPI-6 is highly variable amongst serovars and usually encodes a fimbrial operon (Folkesson et al., 1999), and *pagN*, a virulence gene activated by the PhoPQ two-component system (Gunn et al., 1998, Lambert & Smith, 2009). SPI-7 is found in *S.* Typhi and Paratyphi isolates and encodes a capsular antigen known as Vi encoded on the *viaB* operon (Pickard et al., 2003). SPI-8 through SPI-21 are much more serovar specific than the first seven, often restricted to a single serovar (Hensel, 2004).

Salmonella pathogenicity island one

The first pathogenicity island to be discovered in *Salmonella* was SPI-1, which encodes a T3SS, effectors, and virulence chaperones (Groisman & Ochman, 1993, Mills et al., 1995, Lucas et al., 2000). SPI-1 is approximately 38.8kb, with a G+C content of 45.9%, which is considerably lower than the 52% G+C content of the core genome. It is also inserted between two genes that are adjacent in *E. coli* K12 suggesting a horizontal evolutionary event (McClelland et al., 2001, Mills et al., 1995). The nomenclature for genes encoded by SPI-1 include *inv, prg, org,* and *spa*

genes encoding mostly apparatus proteins of the T3SS, *hil* genes encoding regulators, *sip* genes encoding translocon components, and *sic* genes encoding chaperones (Lostroh & Lee, 2001). Since their discovery, certain proteins encoded in SPI-1 have been shown to have dual functions and do not necessarily fall under their originally designated nomenclature. The *sitABCD* Fe^{2+} and Mn^{2+} uptake system is also encoded on SPI-1, but is not essential for host cell invasion (Janakiraman & Slauch, 2000).

SPI-1 regulation is complex, with at least 14 regulatory inputs involving both environmental signalling and unknown stimuli (Ellermeier & Slauch, 2007). Coordinated transcriptional activation at these promoters must occur to transcribe the many apparatus proteins, virulence chaperones, and effectors of the T3SS. Virulence chaperones aid in the assembly of the T3SS, and help target effectors to the T3SS for secretion. Once assembled, the SPI-1 T3SS injects effectors that facilitate bacterial-mediated phagocytosis. This is mainly mediated by manipulation of the host cell cytoskeleton via actin remodelling effectors (Finlay et al., 1991, Francis et al., 1993, Fu & Galan, 1998, Zhou et al., 1999). Additional effector functions include disruption of tight junctions, guanine nucleotide exchange factor (GEF) mimicking, and inhibition of JAK pathway signalling (Mallo et al., 2008, Liu et al., Boyle et al., 2006). Recently, multiple functions for individual SPI -1 effectors have been discovered at late points during intracellular infection, indicating that they may aid in the function of effectors secreted by the SPI-2 T3SS to enhance bacterial survival within the host (Humphreys et al., 2009, Rodriguez-Escudero et al., 2011).

Salmonella pathogenicity island two

SPI-2 was the second pathogenicity island to be discovered in *S. enterica*. It is approximately 40kb in size, inserted in a *valV* tRNA locus (Shea et al., 1996). The genes in this island encode for a second T3SS and are found only in *S. enterica* serovars, where SPI-2 is the major distinguishing factor between *S. enterica* and *S. bongori*. This island has a G+C content of 44%, suggesting a horizontal transfer event similar to SPI-1 (McClelland et al., 2001). SPI-2 contains 32 genes that contribute to T3SS function, including structural genes for the T3SS apparatus (prefix *ssa*), effectors (*sse*), chaperones which act to assemble translocon machinery or aid in effector secretion (*ssc*), and a few genes with unknown or debated function.

The regulation of genes encoded in SPI-2 is complex involving multiple positive and negative regulators. Perhaps the most important regulator is the SsrAB two-component system encoded within SPI-2 (Deiwick et al., 1999). The SsrAB regulatory system is composed of an environmental sensor kinase SsrA and a response regulator SsrB, whereby SsrB is activated to act as a transcription factor via interaction with and phosphotransfer from SsrA (Fass & Groisman, 2009, Brown et al., 2005). A central theme for SsrB-mediated virulence gene expression regulation is that although encoded in SPI-2, it has adopted regulatory roles for genes encoded elsewhere in the genome (Worley et al., 2000, Coombes et al., 2005a, Coombes et al., 2007). A systematic evaluation of SsrB binding throughout the *S. enterica* genome was conducted that highlighted several genes and gene clusters regulated by SsrB. This study also shed light on new promoters present within SPI-2 (Tomljenovic-Berube et al., 2010). The identification of genes encoded outside of SPI-2 that had been integrated into the SsrB regulon has lead to the conceptualization of the theory of cis-regulatory evolution. In this form of evolution, open reading frames (ORFs) are not mutated under evolutionary pressure, but rather

the regulatory regions themselves change to be incorporated into existing or novel transcriptional regulons. This phenomenon was described for the SsrB-regulated gene *srfN*. This gene is present in both *S. bongori* and *S. enterica*, however in the latter the promoter region has mutated to a sequence that allows SsrB to bind and stimulate transcriptional activation (Osborne et al., 2009).

Additional inputs to SPI-2 regulation include the conserved virulence associated two-component regulatory systems PhoPQ and OmpR-EnvZ, and the transcription factor SlyA (Lee et al., 2000, Bijlsma & Groisman, 2005, Linehan et al., 2005). Negative regulators of SPI-2 gene expression include YdgT, Hha, and the nucleoid DNA binding protein HNS (Duong et al., 2007, Bustamante et al., 2008, Coombes et al., 2005b, Silphaduang et al., 2007). Although the regulatory inputs contributing to T3SS pathogenicity island expression are intricate, our understanding of the precise assembly of the T3SS structure is proving to be equally complex as advancements in structural data and assembly characterization continue.

Type three secretion systems

Widely distributed throughout pathogenic and commensal bacteria, T3SSs are macromolecular secretion systems that form a bridge between the bacterial and host cell cytoplasm (Coombes, 2009). These complexes are formed from over 20, often oligomeric, proteins, and are embedded in the inner, outer, and host cell membranes, making them one of the largest protein complexes known. The function of T3SSs is to receive effector proteins at the proximal end via interaction with sorting platforms and T3SS ATPases and then transport the effector to the host cell cytoplasm. In general, T3SS apparatus proteins are conserved as they travel on pathogenicity

islands or virulence plasmids acting as mobile elements exchanged between pathogens (Juhas et al., 2009, Hall, 2011).

The basal apparatus of the T3SS consists of two proteins that form a heteroligomeric ring in the inner membrane (IM ring) and proteins which form an oligomeric ring in the outer membrane (OM ring). The most studied component of the IM ring is suggested to have either 12 to 20 or 24 monomers and is composed of the PrgK/SsaJ/EscJ/YscJ/MxiJ family of T3SS proteins from SPI-1, SPI-2, EPEC, *Yersinia*, and *Shigella* respectively (Kimbrough & Miller, 2000, Crepin et al., 2005, Yip et al., 2005b, Silva-Herzog et al., 2008, Hodgkinson et al., 2009). The second protein component of the IM ring is composed of the PrgH/SsaD/EscD/YscD/MxiG (from SPI-1, SPI-2, EPEC, *Yersinia*, and *Shigella* respectively) protein family which interacts with the above protein family to form a stable complex (Schraidt et al., 2010, Sanowar et al., 2010). Structural data is available for PrgH and EscJ (SPI-1 and EPEC), however data is not available for two proteins from the same species found in the same T3SS which would further characterize the IM ring interactions (Yip et al., 2005b, Sanowar et al., 2010). The second component of the IM ring also possesses periplasmic motifs that have been suggested to interact with proteins of the OM ring to form a bridge between the two rings (Spreter et al., 2009). Also associated with the IM ring are five apparatus proteins that have yet to be characterized. Electron microscopy data suggests that these proteins localize within the IM ring and may have a role in stabilizing unfolded effectors within the T3SS (Wagner et al., 2010).

The protein components of the OM ring consist of 12-14 monomers of the InvG/SsaC/EscC/YscC/MxiD family (from SPI-1, SPI-2, EPEC, *Yersinia*, and *Shigella* respectively), and have been shown to likely interact with proteins from the IM ring (Spreter et al., 2009, Yip et al., 2005b, Sanowar et al., 2010). Extending from the OM ring is the longest

protein oligomer of the T3SS, the needle filament. The length of the filament varies and is regulated in *Yersinia* by a "ruler protein" termed YscP (Journet et al., 2003). Different YscP lengths determine different needle filament lengths in various species of *Yersinia*; length is also determined based on the α-helical content of YscP (Wagner et al., 2009, Agrain et al., 2005a, Agrain et al., 2005b). This mechanism is also present in the related flagellar system, where FliK controls hook length (Hirano et al., 1994, Mizuno et al., 2011). Both FliK and YscP share weak sequence similarities and the only other identifiable proteins with any similarity are uncharacterized proteins in *Pseudomonas aeruginosa*. This together suggests that control of needle filament length may not be a conserved process, and that additional mechanisms may exist in other species.

The filament structure is composed of a number of repeats of a small, approximately 9-20 kDa, α-helical protein that forms an oligomeric helical-like structure with a 25 Å central channel (Picking et al., 2005, Broz et al., 2007, Blocker et al., 2008). Structural data is currently available for PrgI/MxiH/BsaL of SPI-1, *Shigella flexneri*, and *Burkholderia pseudomallei* (Deane et al., 2006, Blocker et al., 2001, Zhang et al., 2006). Due to their ability to polymerize rapidly, needle monomers are bound by virulence chaperones until they are secreted via the T3SS (Coombes et al., 2003, Quinaud et al., 2005). At the distal end of the needle filament, a complex known as the translocon is assembled for interaction with the host cell membrane. The translocon is often a two-protein ring structure much like the structure of the IM ring. These proteins are secreted from the T3SS and are easily detected in bacterial cell culture supernatant (Coombes et al., 2003, Ruiz-Albert et al., 2003, Zurawski & Stein, 2003).

Secretion of translocon components and effectors is initiated by docking of these substrates, often escorted by virulence chaperones, to the ATPase at the cytoplasmic face of the

T3SS (Akeda & Galan, 2005, Thomas et al., 2005). Recently, a protein complex known as a sorting platform has been implicated in regulating effector and translocon component secretion in response to environmental stimuli (Yu et al., 2010). Although the mechanisms by which the T3SS ATPase and sorting platform interact with the T3SS have not been fully elucidated, studies have shown that proteins known as "c-ring" proteins are necessary for docking of T3SS ATPase (Biemans-Oldehinkel et al., 2011, Yu et al., 2011, Jackson & Plano, 2000, Blaylock et al., 2006).

Recently, advances in the understanding of temporal T3SS assembly have been elucidated by GFP tagging apparatus proteins and examining their localization in different apparatus mutant backgrounds, to assess which proteins are required to assemble before others (Diepold et al., 2010). This study was conducted in *Yersinia enterocolitica*, and involved tracing the localization of the GFP tagged proteins to the membranes of bacterial cells with fluorescence microscopy. Surprisingly, this study's central finding was that assembly of the T3SS occurs in an outside-in order, where YscC is capable of localizing to the outer membrane in the absence of other T3SS components, and IM ring components require the YscC protein for localization. Furthermore, the T3SS ATPase YscN and the c-ring protein YscQ required each other for membrane localization, suggesting that a complex of the two is necessary for association. This complex also required YscK and YscL; previously uncharacterized proteins. Interestingly, assembly was independent of the inner membrane associated YscRSTUV apparatus proteins, indicating they may be central to effector secretion but do not have a scaffold like function for cytoplasmic facing T3SS apparatus proteins. This landmark study is the most detailed description of T3SS assembly, yet there remain many apparatus components that have unknown function and assembly properties.

The cytoplasmic ring structures and sorting platforms of the T3SS

Perhaps one of the most diverse T3SS processes is the docking of effector proteins prior to their secretion to the host cell. The proximal end of the T3SS is one of the most poorly conserved regions, and is the site for effector, virulence chaperone, and sorting platform docking. Breakthrough studies have begun to characterize T3SS ATPase structure and function (Akeda & Galan, 2005, Akeda & Galan, 2004, Zarivach et al., 2007, Imada et al., 2007, Biemans-Oldehinkel et al., 2011), and the requirement of the c-ring for T3SS ATPase association and T3SS function (Biemans-Oldehinkel et al., 2011, Yu et al., 2011, Jackson & Plano, 2000, Blaylock et al., 2006, Fadouloglou et al., 2004, Spaeth et al., 2009). A complex issue that remains unknown is the interaction of sorting platforms with virulence chaperones and the T3SS.

The study of sorting platforms that aid in the temporal regulation of effector secretion is in its infancy. Since the initial identification of a SPI-2 encoded protein, SsaL, as a pH-dependent regulator of effectors and translocon components (Coombes et al., 2004), additional studies have demonstrated that a complex of SsaL, SsaB (formerly known as SpiC), and SsaM acts as a pH-sensitive T3SS plug. At low pH, association of SsaBLM allows for the secretion of translocon units (Figure 1.1). Dissociation at neutral pH subsequently allows for secretion of effectors. Effector secretion is highly active in an *ssaL* mutant, further supporting this concept (Yu et al., 2010). Despite the appealing model that a novel pH-dependent protein complex can act as a hierarchal regulatory input, the SsaBLM complex has not yet been shown to interact with the T3SS and the mechanism for pH detection remains unexplained. The authors hypothesize that a pH sensor protein must be present in order to detect the change in pH within the host cell cytoplasm. This hypothesis is perhaps illogical, as the pH of the SCV would likely be more variable and easily detected by the bacterium. Although several pH dependent two component

regulators associated with T3SS regulation have been identified, these act as transcription factors, not protein complex dissociation agents (Nakayama & Watanabe, 1995, Mondragon et al., 2006, Tremblay et al., 2009, Prost et al., 2007).

Sorting platforms have also been identified for other pathogens harbouring T3SSs. An orthologue of SsaL in EPEC, SepL also controls the regulation of translocon and effector secretion (Kresse et al., 2000, Deng et al., 2005, Wang et al., 2008). SepL has been shown to form a complex composed of SepL, SepD, and CesL, which all have a degree of sequence similarity to the SsaBLM complex encoded within SPI-2 (O'Connell et al., 2004, Younis et al., 2010). Additionally, CesL has been implicated in interacting with Ler, a transcriptional regulator of the locus of enterocyte effacement (LEE) (Singh, 1992). This suggests that the EPEC sorting complex may function as both a genetic regulator and a protein secretion regulator. Another sorting platform is the YopN complex in *Yersinia*. This complex is composed of YopN, its heterodimeric chaperones SycN and YscB, and a regulatory protein termed TyeA (Day & Plano, 1998, Cheng & Schneewind, 2000, Iriarte et al., 1998). It was proposed that the complex docks with the T3SS and prevents secretion of effectors (Cheng et al., 2001). In a landmark study, the crystal structures of YopN associated with its SycN-YscB chaperone complex and also with TyeA were solved (Schubot et al., 2005). The crystal structure of SycN-YscB further supports the hypothesis that YopN binds to the cytosolic face of the T3SS to block effector secretion, as the structure bears striking resemblances to other T3SS virulence chaperones (Stebbins & Galan, 2001, Luo et al., 2001). One difference between the SsaBLM complex and the YopN system is that YopN is secreted, and that this secretion is thought to be the cue for effector secretion, not simply the complex's dissociation (Cheng et al., 2001). MxiC, a potential sorting protein in *Shigella*, shares both sequence and structural similarity to YopN, and is also secreted (Deane et

al., 2008). MxiC is thought to act as a switch for effector secretion, as translocon components are not secreted in a *mxiC* mutant, and effectors have a hyper secretion profile similar to *yopN* and *ssaL* mutants (Botteaux et al., 2009). Interestingly, there are no published attempts at addressing the mechanism of sorting platform association with the T3SS, although further studies will likely provide insight for an interaction with additional T3SS apparatus components.

As T3SS ATPases and the c-ring family of proteins are essential for proper substrate secretion, it is likely that a sorting platform would interact at one or both of these key sites. The c-ring (cytoplasmic ring) comprises a family of proteins that form oligomeric rings at the proximal end of the T3SS and are similar to the majority of oligomeric apparatus proteins associated with T3SSs (Francis et al., 1994, Zhao et al., 1996). Initial research on the c-ring was done in *Shigella*, and demonstrated that Spa33 was associated with the cytoplasmic base of the T3SS and was required for proper effector secretion (Schuch & Maurelli, 2001). Structural data for HrcQb, a c-ring protein of the *Pseudomonas* T3SS, shows a homotetramer structure that likely forms an oligomeric ring by C-terminal motif interactions. HrcQb was also shown to interact with the C-terminal residues of the second c-ring protein, HrcQa, encoded directly upstream of *hrcQb* (Fadouloglou et al., 2004). EscQ, the LEE-encoded c-ring homologue in EPEC, was recently shown to associate with both the apparatus protein EscL and the T3SS ATPase EscN. In addition, EscL was not absolutely required for the interaction between EscN and the c-ring protein EscQ (Biemans-Oldehinkel et al., 2011).

One of the most well characterized c-ring proteins is the SPI-1 encoded SpaO. It has been shown to localize with the T3SS, specifically with apparatus proteins OrgA, OrgB, and the T3SS ATPase InvC. Similar to the role of sorting platform proteins, SpaO binds translocon proteins, acting to ensure that these proteins are secreted prior to effectors; a process that was

dependent on virulence chaperones (Lara-Tejero et al., 2011). This finding is interesting in that previous work by these authors deemed that the ATPase was the interacting partner for virulence chaperone-effector complexes *in vitro*, a result that was not discussed in their latest study. Another pivotal study on c-ring proteins was conducted for the SPI-2 encoded T3SS. Here, *ssaQ* which encodes the c-ring protein was shown to undergo "in-frame initiated translation" resulting in two protein products known as $SsaQ_L$ and $SsaQ_S$. It was also shown that $SsaQ_S$ binds to the C-terminus of $SsaQ_L$ and aids in, but is not essential for, T3SS function (Yu et al., 2011). This genetic mechanism mirrors the *Pseudomonas* T3SS in that two protein products produce the c-ring complex, however in this case tandem genes are used known as $hrcQ_A$ and $hrcQ_B$ (Fadouloglou et al., 2004, Fadouloglou et al., 2009). The highly diverse mechanisms used by c-ring proteins and sorting platforms across different species is proving to be a complicated yet rapidly expanding field. The dogmatic view that T3SSs are a highly conserved functioning unit, has been challenged based on the divergent strategies observed for virulence chaperone and effector docking at the cytoplasmic face of the T3SS, possibly to accommodate divergent effector strategies in different pathogens.

The T3SS ATPases

T3SS ATPases are a conserved family of proteins found at the base of the T3SS and are involved in effector-chaperone complex binding. These enzymes are highly conserved despite being located in a region of the T3SS with low conservation. The general theme for effector recognition and interaction at the T3SS is that different sorting platform and c-ring strategies are required to handle different virulence chaperone and effector repertoires, therefore it is surprising that the T3SS ATPases are so well conserved. The structures of T3SS ATPases and, to a lesser

extent, their primary amino acid sequence are similar to the hexameric F_1F_0 α and β subunits of the ATP synthase which is required to manufacture ATP, and may be the evolutionary ancestor of T3SS ATPases.

One of the first characterized T3SS ATPases was YscN from *Yersinia*, which was shown to contain the Walker boxes necessary for ATP interaction (Walker et al., 1982). Mutation of the Walker box A resulted in an inability to secrete the *Yesinia* effectors, known as *Yersinia* outer proteins (Yops) (Woestyn et al., 1994). YscN interacts with YscL, a regulator of secretion, which can also inhibit ATPase activity of YscN *in vitro* (Blaylock et al., 2006). The authors of this study also showed that secretion of the effector YopR is dependent on its interaction with YscN, and that YopR secretion could be inhibited by the attachment of tightly folded protein domains that possibly inhibited interaction with YscN (Sorg et al., 2006). The *Chlamydia pneumoniae* T3SS ATPase CdsN has also been characterized. CdsN was shown to interact CdsD, CdsL, CdsQ, and CopN, four putative structural components of the *C. pneumoniae* T3SS. It also possesses ATPase activity and oligomerizes into dimeric and trimeric forms *in vitro* (Stone et al., 2008). In a follow up study, a defined interaction between CdsN and the putative T3SS component CdsL was mapped and characterized. This characterization led to the development of a CdsN peptide mimic that would interact with the CdsN complex, possibly preventing CdsN oligomerization and ultimately preventing invasion of *C. pneumoniae* into epithelial cells (Stone et al., 2011). The *Pseudomonas* T3SS ATPase HrcN forms monomers, hexamers, and dodecomers *in vitro*. The hexameric isoform was found to be the predominant form when associated with membrane fractions, although ATPase activity was highly increased with the dodecomer form *in vitro* (Pozidis et al., 2003). Later, the same group was able to visualize the HrcN dodecamer by cryo-electron microscopy, showing that it is likely composed

of two hexameric ring structures that interact via their C-terminal regions (Muller et al., 2006). EscN, the well-characterized LEE-encoded T3SS ATPase from EPEC was first shown to be necessary for T3SS function and served as a docking point for the effector Tir in conjunction with its virulence chaperone CesT (Gauthier et al., 2003, Gauthier & Finlay, 2003). Oligomerization studies with EscN showed that a hexamer is formed *in vitro* that also possesses ATPase activity (Andrade et al., 2007). A 1.8Å resolution crystal structure of EscN is the only T3SS ATPase structure to be published. A 102 amino acid N-terminal truncation and a V393P mutation was required to obtain soluble protein. The structure of EscN is made up of a central, nine parallel β-strand scaffold surrounded by seven α-helices and a C-terminal motif projecting off the end of the scaffold containing five α-helices. The structure was modelled into a hexamer, showing a distinct arginine finger motif for the binding of ATP between monomers. Mutation of this residue abolished both ATPase activity and effector secretion, confirming its critical role in EscN function (Zarivach et al., 2007).

While EscN remains the only T3SS ATPase with a published crystal structure, the most biochemically characterized ATPase is the SPI-1 encoded InvC. It contains the conserved Walker boxes indicative of an ATPase, which when mutated abolish ATPase activity and invasion of epithelial cells (Eichelberg et al., 1994). A functional dissection of mofits important for several InvC characteristics was also conducted. Although multiple residues throughout the protein are necessary for effector secretion and epithelial cell invasion, a residue in the N-terminus was found necessary for association with the membrane fraction. No mutations were found that would cause dissociation from the T3SS apparatus protein OrgB, this is contradictory to the finding that an N-terminal region is required for membrane localization, as an interaction with OrgB would lead to localization within the membrane fraction (Akeda & Galan, 2004).

After these properties were identified, the same group expanded on their findings to characterize chaperone-effector complex interactions with InvC *in vitro*. The virulence chaperone SicP and its effector substrate SptP, were found to bind InvC in an ATP independent manner, however while dissociation and release of the chaperone from the effector occurred in the presence of InvC, it was dependent on the ATPase activity of InvC. The release of the effector–chaperone complex also resulted in the unfolding of the effector, a process necessary for secretion through the T3SS (Akeda & Galan, 2005). The T3SS ATPase family is essential for T3SS function, and while the mechanisms of their integration into the cytoplasmic face has begun to be elucidated, many issues as to precisely how sorting platforms, c-rings, and the functional T3SS ATPase enzymes co-ordinate effector secretion at the base of the T3SS still remain uncertain.

Translocons of the T3SS

The translocon components of the T3SS are based at the distal end of the needle filament and are responsible for insertion of the T3SS into the host cell membrane, in order to complete the bridge between bacterial and host cell cytosols. Information from different species and systems has shown that while the general function of translocons is conserved, their formation, and attachment to the needle filament vary. Generally, two hydrophobic proteins form a ring structure embedded in the host cell membrane, while one hydrophilic protein acts a platform and facilitates the attachment of the ring to the elongated needle filament (Figure 1.2A) (Mueller et al., 2008, Sato & Frank, 2011, Blocker et al., 2008, Sarker et al., 1998).

The *Shigella* translocon system is composed of the hydrophobic IpaB and IpaC proteins that are joined to the MxiH needle filament by the hydrophilic IpaD (Blocker et al., 1999, De Geyter et al., 2000, Guichon et al., 2001, Picking et al., 2005, Espina et al., 2006, Veenendaal et

al., 2007). Assembly of the translocon is mediated by the virulence chaperone IpgC that enables secretion of IpaB and IpaC (Page et al., 1999). Structural data for IpaD and the *Yersinia* orthologue LcrV show that common elongated α-helical motifs within the hydrophobic platform protein likely interact with the α-helical regions of the needle filament, while the N-terminal motifs likely support the translocon rings (Johnson et al., 2007, Derewenda et al., 2004). Based on the modelled configuration of the needle filament, the LcrV platform protein is suggested to form a homopentamer, keeping C-terminal α-helices in-line with the projecting α-helices from the *Yersinia* needle protein YscF (Deane et al., 2006, Holmstrom et al., 2001). The translocon proteins from *Yersinia* (YopB and YopD) and *Shigella* (IpaB and IpaC) show an unusually high sequence and structural similarity as conservation scores for translocon proteins are generally low amongst T3SS (Hakansson et al., 1993, Mueller et al., 2005, Sani et al., 2007). YopB and YopD were shown to be sufficient to puncture cell membranes by a study examining the infection of cells with a *Yersinia* strain lacking secreted effectors, but possessing a functional translocon. It was also found that removal of the translocon resulted in a decrease in host cell membrane permeability (Neyt & Cornelis, 1999a). In addition, although *Yersinia yopB* and *yopD* mutants can readily secrete effectors into bacterial culture supernatants, they are unable to translocate effectors into the host cell cytoplasm (Olsson et al., 2004).

Although the translocon proteins remain similar in structure and function across T3SSs, the hydrophilic translocon platform proteins vary in their length and structure. After imaging studies for IpaD and LcrV of *Shigella* and *Yersinia* revealed that a distinctly localized platform protein existed for the translocon, a different mechanism was discovered in EPEC. The translocon platform protein in EPEC is EspA, although it is also filamentous, similar to needle filament structures such as YscF and MxiH (Figure 1.2B) (Yip et al., 2005a, Wang et al., 2006,

Daniell et al., 2003, Sekiya et al., 2001). This was discovered by imaging the needle filament at the point of translocon interaction in wild type and an *espA* mutant background, and determining that the needle filament had a sheath-like structure composed of both EspA and the needle filament EscF (Sekiya et al., 2001). A classification scheme was proposed that the translocon platform proteins of T3SSs fall under the category of either a single (IpaD and LcrV) or filamentous type (EspA). Despite having a unique filamentous type platform protein of EspA, EPEC still possesses the well-conserved translocon protein pair termed EspB and EspD (Wachter et al., 1999, Hartland et al., 2000, Luo & Donnenberg).

The SPI-1 T3SS distal end is composed of a single needle filament, PrgI, and the platform protein, SipD, which stabilize the two hydrophobic translocon components SipB and SipC. PrgI was first identified as the needle filament by its visualization with electron microscopy using antibodies directed to PrgI (Kubori et al., 2000, Kimbrough & Miller, 2000). The translocon platform protein SipD was identified based on homology studies with *Shigella* IpaD (Kaniga et al., 1995a). Recently, studies have presented structural data for SipD, and SipD in complex with PrgI. SipD has three motifs: a central motif composed of two α-helices supporting a small N-terminal α-helical motif at one end, and a globular, more complex motif composed of two β-sheets joined by α-helices at the other (Chatterjee et al., 2011, Lunelli et al., 2011). The SipD-PrgI co-crystal revealed an interaction between the N-terminal α-helix of PrgI and the 4^{th} α-helix of SipD (Lunelli et al., 2011). When modelled together as the functional *in vivo* ring complex, a pentamer of SipD with the N-terminal motif bent away from the central channel sits on top of a pentamer of PrgI. In support of this model, the approximated channel diameter of 25Å strongly correlated to other proposed models (Blocker et al., 2008).

While no structural data has been published for the translocon components of the SPI-2 encoded T3SS, candidate proteins were identified through homology studies and biochemical analysis. SseB, SseC, and SseD were initially characterized as being surface expressed and required for effector translocation, but not secretion (Hensel et al., 1998, Nikolaus et al., 2001). When a subsequent study attempted to define functional motifs in SseB and SseD it was found that mutations in any of the residues examined resulted in an inability to translocate effectors (Holzer & Hensel, 2011).

A thorough understanding of T3SSs requires further analysis of the precise formation of the filamentous platform proteins such as EspA and their interactions with the translocon components. In order to determine the exact composition of the needle filament, additional studies should focus on the characterization of EscF and the putative SPI-2 T3SS filaments SsaG and SsaH. Furthermore, little is known about the mechanisms of needle filament insertion or extension from the T3SS basal body or the forces driving effector secretion, and addressing such questions should be the focus of future work.

Effector functions

The goal of the T3SS is to bridge bacterial and host cytosol, and allow for passage of bacterial effector proteins to the host. The number of characterized effectors is vast, found in symbionts to pathogens, performing a number of functions from histone acetylation to modulating host cell migration (Mukherjee et al., 2006, McLaughlin et al., 2009). The composition of effectors is highly diverse, although they may be grouped based on consensus sequences and host modification function. An N-terminal secretion signal is often found on effectors, which when added to other proteins, enables secretion of those heterologous substrates in a T3SS-dependent

manner (Munera et al., 2011). Prediction of a secretion signal motif is difficult; only recently have weak consensus motifs been computationally generated (Lower & Schneider, 2009, McDermott et al., 2010, Samudrala et al., 2009). Another motif includes the WXXXE motif common to a large family of effectors that act as guanine nucleotide exchange factors (GEFs) within the host cell (Alto et al., 2006, Bulgin et al., 2011, Raymond et al., 2011, Orchard & Alto, 2011). Additional conserved motifs found in effectors include nuclear localization signals (NLS), mitochondrial localization signals, and palmitoylation sites (Bartfeld et al., 2009, Hicks et al., 2011, Kurushima et al., 2011).

As effectors are identified and functionally characterized, several recurring themes are noticeable. Certain effector groups may share sequence similarity, structural correlation, and host target proteins. One of the largest groups of effectors, based on host protein interaction are GEF effectors. This group of effectors mimic eukaryotic GEFs that interact with GTPases to release GDP to allow GTP to bind. This family has been termed the WXXXE family as they all contain a Trp-X-X-X-Glu motif. An example of a GEF effector is the Map protein encoded within the LEE of EPEC. This protein disrupts cell morphology through cytoskeletal remodelling by binding to the Cdc42 GTPase (Huang et al., 2009, Jepson et al., 2003). An additional group contains effectors that covalently modify host proteins through phosphorylation or acetylation. The *Shigella* OspG effector phosphorylates UbcH5 that prevents both the degradation of IκBα and the activation of NF-κB transcription, inhibiting an inflammatory response (Kim et al., 2005).

The family of ubiquitination modulation effectors exert their function at several sites throughout the ubiquitination pathway. The process of uquitination involves the addition of the 9 KDa protein ubiquitin to proteins often destined for the proteasome or in some cases other

regions of the eukaryotic cell (Seufert & Jentsch, 1992). The most targeted ubiquitin pathway in eukaryotes is the poly-ubiquitination of IκBα. Effector inhibition of the poly-ubiquitination of IκBα prevents the release of NF-κB and the induction of the pro-inflammatory response. This function was first described for the effector YopJ in *Yersinia* (Orth et al., 2000). Perhaps one of the most easily characterized effector functions is the polymerization of host cytoskeleton components, detectable with phalloidin staining of filamentous actin, and readily observed at the point of contact for EPEC adhesion to host cells (Knutton et al., 1989, Jerse et al., 1990, Kenny et al., 1997). Several other pathogens use similar effector functions to polymerize and manipulate the host cytoskeleton for either attachment or invasion (Haglund & Welch, 2011). While the majority of effectors may fall into one of the above categories, evolution has produced countless additional effectors with other astonishingly intricate methods for host cell modification.

The temporal regulation of transcription and secretion of effectors within the bacterial cell is complex, and recently their temporal regulation within host cells has been addressed. As the process of host cell infection progresses, different effector functions are needed by the infecting bacterium. In the case of *S. enterica* infections, effectors are first required to enact bacterial mediated endocytosis of epithelial cells, followed by disruption of tight junctions, dampening of host cell inflammatory response, establishing and maintaining an SCV, and promoting centrifugal movement of the SCV (Szeto et al., 2009). The coordination of effector function during all of these processes occurs by two identified methods. First, *S. enterica* possesses two T3SS, with the SPI-1 encoded T3SS assembled prior to host cell contact, and the SPI-2 T3SS used for effector secretion from the SCV (Altier, 2005). Effectors may be secreted through one or both of these systems depending on the temporal requirement of their function.

Additionally, a major factor involved in regulating effector function within the cell is the half-life of effectors within the host cell cytoplasm. An example of this regulation is seen for the SPI-1 associated effectors SopE and SptP. Although both effectors are secreted at the same time, the half-life of SopE is much shorter than SptP (Kubori & Galan, 2003). The process of effector secretion regulation in the bacterial cytosol, and effector function regulation within the host cell illustrates the complexity involved in the precise regulation of effector function during infection.

Effectors secreted through the SPI-1 encoded T3SS

Effectors secreted through the SPI-1 encoded T3SS were originally thought to be responsible solely for actin polymerization during the invasion of *Salmonella* into host cells. As data has accumulated for SPI-1 associated effectors, it appears as though half of the ten identified effectors participate in actin remodelling, while the other half produce effects such as the reversal of actin polymerization and inhibition of host signalling. Both SipA and SipC stimulate the polymerization of filamentous actin by binding to actin directly (Zhou et al., 1999, Hayward & Koronakis, 1999). SopE, SopB, and SopE2 cause actin polymerization through GEF function at the cytoskeletal remodelers Cdc42 and Rac1 (Lamarche et al., 1996, Rudolph et al., 1999, Stender et al., 2000, Rodriguez-Escudero et al., 2006). This activation of Cdc42 and Rac1 also has a degenerative effect on tight junctions, possibly causing further permeability and access to the intestinal lamina propria (Rojas et al., 2001, Jou et al., 1998). The additional five SPI-1 associated effectors include SopD, a protein that is able to activate the host protein Akt, which inhibits apoptosis (Knodler et al., 2005). SopD requires SopB dependent phosphorylation to gain its membrane localization (Bakowski et al., 2007). Although no function has yet been determined for the SopA effector, structural analysis shows similarity to E3 ubiquitin ligases,

indicating it likely has a role in host signalling disruption (Diao et al., 2008). SipB induces apoptosis in macrophages by binding and activating caspase-1, although how this is of benefit to the virulence strategy of *Salmonella* is not currently known (Santos et al., 2001, Hersh et al., 1999). Interestingly, the AvrA effector must be phosphorylated by the ERK pathway prior to its inhibition of the JNK pathway at MKK7, suggesting that inflammatory responses are required for activation of AvrA, so that these same responses may then be inhibited (Du & Galan, 2009). SptP is an effector secreted through the SPI-1 encoded T3SS with diverse functions within the cell. It was originally characterized as a GTPase activating protein (GAP) protein (opposite function as GEF) acting to return the actin cytoskeleton to its normal state after *Salmonella* mediated remodelling (Kaniga et al., 1996, Fu & Galan, 1998, Fu & Galan, 1999). However, SptP has recently been shown to have dual effector functions, with activity derived from a C-terminal domain that dephosphorylates and thus deactivates the host valosin-containing protein (VCP) protein. VCP is involved in phagosome-lysosome fusion, and its inhibition enhances bacterial survival within the SCV (Humphreys et al., 2009). This second function for SptP is clearly one that aids in intracellular survival, a task normally denoted to SPI-2 associated effectors, suggesting more of an overlap between the two systems then previously appreciated.

Effectors secreted through the SPI-2 encoded T3SS

In general, effectors secreted through the SPI-2 encoded T3SS are responsible for ensuring bacterial survival within the host cell. The main function of this group of effectors is maintenance of the SCV and contributing to the formation of *Salmonella* induced filaments (SIFs). These projections emanate from the SCV and are believed to help prevent SCV fusion with lysosomes (Schroeder et al., 2011b). Recently, their composition has been documented as

containing lysosomal protein, Golgi proteins, and sorting proteins termed nexins (Braun et al., 2011, Bujny et al., 2008). The two effectors encoded on SPI-2, SseF and SseG, are found on, and contribute to SIF formation, although a precise mechanism for this is unknown (Kuhle & Hensel, 2002, Hansen-Wester et al., 2002). The set of three highly conserved effectors known as SseK1, SseK2, and SseK3 have their corresponding genes distributed throughout the genome. While no function has been elucidated for these effectors, they have sequence similarity to NleB effectors encoded within EPEC that are responsible for inhibiting NF-κB activation, and mutating combinations of them causes a virulence defect in murine infections (Newton et al., 2010, Brown et al., 2011). Other secreted effectors with unknown functions include the SteA, SteB, and SteC set of effectors as well as SifB, and PipB (Geddes et al., 2005, Miao & Miller, 2000, Knodler et al., 2003). Two effectors that share sequence similarities are SspH1 and SspH2, although a function has been determined for only SspH1, which ubiquitinates PKN1 resulting in inhibition NF-κB activation (Haraga & Miller, 2003, Rohde et al., 2007). SspH2 shares sequence similarity with SspH1, has structural motifs resembling an E3 ubiquitin ligase, is localized to the host cell membrane upon host palmitoylation, and is required for full virulence in a mouse infection, however no exact mechanism of host interaction or modification has been determined for SspH2 (Hicks et al., 2011, Quezada et al., 2009, Miao et al., 1999, Miao et al., 2003). The SseL effector also modifies the host ubiquitination pathway, through its de-ubiquitination of IκBα leading to sequestering of NF-κB in the cytosol (Le Negrate et al., 2008, Rytkonen et al., 2007, Coombes et al., 2007). This may also result in the accumulation of lipid droplets in the host cell, suggesting that lipid metabolism signalling is also affected (Arena et al., 2011). The GogB effector is secreted through both the SPI-1 and SPI-2 encoded T3SSs, despite being regulated by the SPI-2 encoded SsrB transcription factor (Coombes et al., 2005a). The

function of GogB in the host cell is the inhibition of IκBκ degradation through its interaction with the SCF (Skp, Cullin, F-box) containing ubiquitin ligase complex proteins FBXO22 and Skp1, resulting in NF-κB sequestering (Pilar et al. 2011, submitted). An additional ubiquitin pathway interfering effector is SlrP, which is also secreted from both the SPI-1 and SPI-2 encoded T3SSs. SlrP acts as an E3 ubiquitin ligase for the host protein thioredoxin causing its degradation and an increase in apoptosis (Bernal-Bayard & Ramos-Morales, 2009). Interestingly, SlrP has another host protein target Erdj3, which is located in the endoplasmic reticulum, possibly leading to an increase in mis-folded proteins and eventually the onset of apoptosis (Bernal-Bayard et al., 2011). SseI binds to bind the host protein IQGAP1, interrupting normal cell migration, which is believed to prevent clearance of *S. enterica* (McLaughlin et al., 2009). Recently this function was shown to be dependent on host palmitoylation of SseI, to allow membrane localization for the interaction with IQGAP1 (Hicks et al., 2011).

The most complex group of SPI-2 associated effectors is the SopD2, SifA, PipB2, and SseJ set of SCV localized proteins. These effectors act to temporally regulate the position of the SCV within the host cell. During early infection time points, SifA localizes to the SCV, and binds to the host protein SKIP, which inhibits the recruitment of the microtubule motor kinesin to the SCV (Boucrot et al., 2005, Beuzon et al., 2000). This keeps the SCV positioned at the centre of the cell next to the nucleus (Ramsden et al., 2007). It is also believed that SseJ has a complementary effect on SifA, as SseJ co-localizes with SifA and is required for SCV maintenance (Ruiz-Albert et al., 2002). Interestingly, at later stages of infection this process is reversed by PipB2 and SopD2 effector function. PipB2 is responsible for the localization of kinesin to the SCV, a function that is antagonistic to SifA (Henry et al., 2006). This recruitment of kinesin results in plus end microtubule mediated movement of the SCV towards the host cell

membrane (Szeto et al., 2009). It is believed that SopD2 plays a role in this movement due to its co-localization with PipB2, although no mechanism for a PipB2 complimentary role has been described (Schroeder et al., 2011a). Furthermore, an additional antagonistic effect on the properties of PipB2 and SopD2 is the recruitment of myosin II to the SCV, which would act to drive the SCV in a minus end direct movement. This has been suggested as an additional role for the SPI-1 encoded T3SS secreted effector SopB, adding yet another layer of control to SCV dynamics (Wasylnka et al., 2008). Taken together, the over 20 effector proteins secreted through the SPI-2 encoded T3SS have a dramatic impact on host cell function. Although one area that remains unclear is their association with the cytoplasmic face of the T3SS and whether or not they require specialized virulence chaperones for delivery.

Virulence chaperones

Chaperones are often essential proteins that aid other proteins with functions such as obtaining initial folded state, targeting to proper cellular localization, and degradation. In bacteria, the most well characterized chaperones are the housekeeping proteins of the DnaK and GroEL/GroES family. These proteins are normally present during protein synthesis and undergo ATP dependent conformational changes during the folding of other proteins (Todd et al., 1994, Gragerov et al., 1992). More specialized chaperones known as virulence chaperones have major roles in bacterial pathogenesis. First identified by Wattiau and Cornelis in *Yersinia*, several virulence chaperones have since been characterized in species with T3SSs (Wattiau & Cornelis, 1993). Virulence chaperones act to direct effectors, translocon components, and needle apparatus proteins to the cytoplasmic face of the T3SS for secretion, as well as stabilizing and preventing premature aggregation of substrates in the bacterial cytosol. As the list of virulence

chaperones continues to grow, a classification scheme has been adopted based on chaperone substrates. Class I virulence chaperones are responsible for aiding the secretion of effectors, and may be further classified as class IA for one effector substrate, and class IB for multiple. Class II bind translocon components, and class III bind apparatus components of the T3SS (Cornelis & Van Gijsegem, 2000, Parsot et al., 2003). Class III virulence chaperones are the smallest class, and are chaperones for needle filament proteins such as PscF in *Pseudomonas*. The PscE and PscG virulence chaperone complex is responsible for binding PscF in the bacterial cytosol and is required for its secretion. The virulence chaperones are also likely present to inhibit polymerization of PscF prior to secretion (Quinaud et al., 2005). The crystal structure of PscE and PscG bound to a short PscF peptide has also been determined. The heterodimer of PscE and PscG is bound to the PscF peptide predominately at PscG, suggesting that PscE may be involved in modification of PscG to enable PscF binding (Quinaud et al., 2007).

The class II virulence chaperones have been characterized in a number of species and possess sequence similarities and a strong structural correlation. The crescent shaped, α-helical structure of SycD, the virulence chaperone for the YopB and YopD components of the *Yersinia* translocon, is indicative of this chaperone class (Buttner et al., 2008, Neyt & Cornelis, 1999b). The IpgC virulence chaperone for the *Shigella* translocon components IpaB and IpaD mirrors this structure with the same crescent shape composed solely of α-helices, and a similar amino acid sequence (Lunelli et al., 2009, Page et al., 1999). The co-crystal of IpgC and an N-terminal peptide of its substrate IpaB showed that the translocon substrate was held within the concave side of the chaperone (Figure 1.3) (Lunelli et al., 2009). The class II virulence chaperone repertoire for EPEC is complex, and includes CesD, CesD2, CesAB, and CesA2. CesD is required for the secretion of EspB and EspD, the two translocon components of the LEE encoded

T3SS, but a protein interaction can only be found with EspD (Wainwright & Kaper, 1998). CesD2 was also identified as a virulence chaperone for EspD, but not for EspB (Wainwright & Kaper, 1998). The CesAB virulence chaperone (also known as CesA) may fall under either class II or III as its substrate EspA is filamentous and may act as both a translocon platform protein and contribute to the needle filament with EspF (Creasey et al., 2003). Additionally, CesA2 is responsible for the secretion of EspA (Su et al., 2008). Structural data is available for CesAB; it has a similar structure to its EspA substrate, composed of three α-helices that surround the entire length of the two central α-helices of EspA (Yip et al., 2005a). Interestingly, the redundancy seen with the two chaperone sets (CesAB-CesA2, and CesD-CesD2) in this system is not found with any other known T3SS. There is no structural data for additional class II chaperones, however sequence and structural prediction similarities between identified chaperones exist. The class II virulence chaperone associated with the *Pseudomonas* T3SS is PcrH which forms soluble complexes with translocon proteins PopB and PopD, and is required for their secretion (Schoehn et al., 2003, Broms et al., 2003). The SPI-1 encoded virulence chaperone SicA is required for the secretion and intracellular stability of the translocon components SipB and SipC (Kaniga et al., 1995b, Tucker & Galan, 2000). The SPI-2 encoded virulence chaperone SseA is responsible for the secretion of SseB and SseD (Zurawski & Stein, 2003, Ruiz-Albert et al., 2003, Coombes et al., 2003, Zurawski & Stein, 2004), however this chaperone may have a similar classification to CesAB, as based on bioinformatic analysis, SseB may function as a filamentous translocon platform contributing to the needle filament. Not only do class II virulence chaperones bind, stabilize, and promote substrate secretion, but they have also been implicated in further virulence roles within the cell.

As mentioned previously, the regulation of T3SS-encoded genes is often complex, composed of both regulators found within the mobile PAI or virulence plasmids in which they are encoded, and those encoded elsewhere in the genome. A further layer of complexity has been observed for virulence chaperone associated gene regulation. This is normally attributed to the substrate's effect on regulation complexes, and the ability for the virulence chaperone to increase substrate stability or sequester it. Examples of this phenomenon occur for the SPI-1 encoded virulence chaperone SicA and its regulator substrate InvF. The DNA binding transcriptional regulator InvF is encoded within SPI-1 and is required for transcription of SPI-1 encoded genes (Kaniga et al., 1994, Darwin & Miller, 1999). Evidence suggests that SicA has a role in regulation of SPI-1 promoters, as *sicA* mutants showed lack of SPI-1 promoter expression (Darwin & Miller, 2000, Tucker & Galan, 2000). In an attempt to explain how SicA is responsible for InvF like function, a protein interaction was found between SicA and InvF, although stability of InvF was not affected by SicA (Darwin & Miller, 2001). The regulatory mechanism of SicA is unknown, although a hypothesis is that an InvF-SicA complex may be able to bind with other regulatory complexes, that InvF alone would not be able to, in order to initiate transcription. This system of class II virulence chaperone and regulator effect on transcription is mirrored in *Shigella* with MixE and IpgC, homologues of InvF and SicA respectively (Mavris et al., 2002). An additional virulence chaperone implicated in transcriptional regulation is SycD (also known as LcrH) of *Yersinia*. The translocon component YopD was first identified as having an effect on gene regulation at promoters within the *Yersinia* virulence plasmid when a mutant strain for *yopD* constitutively expressed *yop* effector genes (Williams & Straley, 1998). Later, a role for SycD in stabilizing YopD, and therefore participating in the regulation of *yop* genes was identified (Francis et al., 2001). A further study

produced the controversial theory that a YopD/SycD complex binds to *yopQ* mRNA in order to allow its translation (Anderson et al., 2002). This finding was challenged by data suggesting that a YopD/SycD complex regulates the availability of the transcription factor LcrQ to bind DNA at virulence plasmid promoters (Olsson et al., 2004). The ability of virulence chaperones to regulate transcription with their substrate counter parts expands the properties of virulence chaperones and their effects on the overall virulence strategy of pathogens.

By far the most characterized and prevalent class of virulence chaperones fall under class I. The first class I virulence chaperone to be identified was SycE encoded in *Yersinia*. This protein was deemed a chaperone for an effector as a SycE mutant strain showed reduced secretion of the YopE effector, however YopE expression and the secretion of additional effectors was unaffected (Wattiau & Cornelis, 1993). Since this discovery, multiple effector chaperones have been identified, with common properties emerging, useful for the identification of additional chaperones. In general, class I virulence chaperones have an acidic isoelectric point (pI), are small in size (approximately 20 kDa or less), and function as homodimers adopting a horseshoe like structure (Figure 1.4). Class IA chaperones are often encoded directly adjacent to their substrates as with the SPI-1 encoded SicP chaperone and its substrate SptP, while class IB may be more sporadically distributed such as with *Shigella* chaperone Spa15 (Stebbins & Galan, 2001, Page et al., 2002). The central function of class I virulence chaperones is trafficking of effectors to the T3SS for secretion, although their function may not be essential, with only a reduction in secretion and not a complete abolishment seen in with some chaperone mutants (Cheng et al., 1997). Additionally, there may not be a requirement for a virulence chaperone pairing with every identified effector. In order to understand why chaperones are required for some effectors, a closer look at motifs within effectors is required. Since the discovery of a

secretion signal on *S. enterica* effectors, attempts to identify a consensus sequence for other organisms have been difficult (Miao & Miller, 2000). The signal is required for proper secretion of a number of effectors, but so too is another domain termed the chaperone binding domain (CBD) (Woestyn et al., 1996). Together, the secretion signal and CBD are required for secretion of some effectors, however their coding is generally in the same area at the N-terminus, so upon chaperone binding, the secretion signal topology may be affected (Birtalan et al., 2002). This may be a cue for secretion, in that the secretion signal is exposed by the binding of the chaperone, and docking with a sorting platform or other structure of the T3SS is enabled.

The structure of class I virulence chaperones from several species is known. As mentioned previously, despite weak sequence similarity, the overall structure of this class of virulence chaperones is surprisingly well conserved. In a comparative study, the crystal structures of virulence chaperone CesT encoded in the LEE of EPEC, and the SPI-1 encoded SigE were determined. Both possess very similar horseshoe-like structures, with an α-β-β-β-α-β-β-α topology and the only major difference being an extra β-strand at the C-terminus of CesT. The acidic pI for both proteins is due to large negatively charged patches found across each protein, thought to be effector or T3SS apparatus interaction sites (Luo et al., 2001). The *Yersinia* virulence chaperone SycE adopts the same shape as CesT and SigE (Trame & McKay, 2003). Later, the structure of SycE bound to YopE was determined using NMR, showing YopE bound across the face of the protein at negatively charged patches (Rodgers et al., 2008). In a co-crystal strategy, the structure of the secreted regulatory protein YopN along with both of its virulence chaperones, YscB and SycN was determined. This triple protein structure is the only known heterodimeric class I virulence chaperone structure. Both YscB and SycN formed the typical horseshoe-like structure with YopN bound across the face of the protein at negatively

charged patches. Additionally, a model for binding of the second regulatory protein, TyeA was determined in the same publication (Schubot et al., 2005). This protein complex raises the issue of the significance of a virulence chaperone binding a regulatory protein and causing its association with the T3SS. An attractive hypothesis is that a translational/translocation strategy whereby proteins are translated directly into the T3SS apparatus exists. This method would mirror the eukaryotic translation of proteins at the endoplasmic reticulum. Another co-crystal strategy yielded the structure of the SPI-1 encoded SicP bound to its effector substrate SptP. Here the typical horseshoe structure is seen however it is composed of a dimer of dimers, not a simple dimeric structure seen with other class I virulence chaperones. Additionally, a detailed presentation of two SptP effector substrate molecules binding to the SicP complex is given, where three SicP molecules are required to bind one highly unfolded effector molecule (Stebbins & Galan, 2001). Class IB virulence chaperones have been crystallized as well, however only two are available. The multi-substrate binding Spa15 virulence chaperone from *Shigella* adopts a similar horseshoe-like structure although the organization is somewhat different from virulence chaperones such as SycE. In a comparison between SycE and Spa15, the authors illustrate that a much more bent complimentary helix-dimer interaction site exists, however the overall shape of the dimers are similar, with the characteristic negative and hydrophobic patches present across the molecule surface (van Eerde et al., 2004). The structure of the SPI-1 encoded InvB which is a chaperone for SipA, SopA, SopE, and SopE2 was determined, and it possesses the conserved horseshoe-like structure. In this study, a small N-terminal portion of SipA was displaced and unfolded in order to bind with InvB (Lilic et al., 2006). With structural data available for all four identified class I virulence chaperones for SPI-1 associated effector interactions, and a chaperone

effector relationship determined for eight of the ten canonical effectors, few unknowns remain for the SPI-1 associated virulence chaperone network.

With over twenty SPI-2 associated effectors known, there is only one class IA virulence chaperone identified. This is the SPI-2 encoded SscB, responsible for interaction with and the secretion of SseF (Dai & Zhou, 2004). The remainder of the SPI-2 associated effectors likely require a virulence chaperone, however no additional chaperones have been suggested, identified, or characterized.

Purpose and goals of the present study

The purpose of the present study is the identification and characterization of class I and II virulence chaperones necessary for secretion of SPI-2 associated substrates. There is a clear knowledge gap existing for the mechanisms used to enable secretion of SPI-2 associated effectors. As these effector functions are critical for the virulence strategy of *S.* enterica, an understanding of their secretion is needed. Additionally, the virulence chaperone for the translocon component SseC remains unknown despite chaperone pairs identified for the additional translocon component SseD and the putative filamentous translocon platform protein SseB. A goal of this study is the identification of the SseC virulence chaperone. Furthermore, the properties of the SPI-2 encoded T3SS ATPase SsaN will be examined to determine how the SPI-2 virulence chaperone network potentially interacts with the T3SS. Not only are virulence chaperones essential for secretion of some effectors, but so too are the apparatus proteins of the T3SS, including the T3SS ATPase enzymes. As these proteins are likely necessary for T3SS function and *S. enterica* pathogenesis, their properties and mechanism of action should be documented to further understand the cell biology of *S. enterica*.

The specific goals of this study are highlighted in the following three chapters:

1. Structural and biochemical characterization of SrcA, a multi-cargo type III secretion chaperone in *Salmonella* required for pathogenic association with a host.
    - This study identified SrcA as a novel class IB virulence chaperone. The structure of SrcA was determined as well as identifying its effector substrates SseL and PipB2.

2. The *Salmonella* pathogenicity island-two encoded virulence chaperone SscA and the translocon component SseC are required for virulence and regulation of SPI-2 encoded genes in *Salmonella enterica.*
    - SscA, a class II virulence chaperone for the translocon component SseC was identified. An interaction between the two proteins and the requirement of SscA for the secretion of SseC was characterized, as well as a transcriptional regulatory role for SseC.

3. Functional and structural characterization of the *Salmonella* pathogenicity island-2 encoded type three secretion system ATPase SsaN.
    - The structure of the SPI-2 encoded T3SS ATPase SsaN was determined, as well as characterizing functional regions of SsaN.

The hypothesis that SrcA is a virulence chaperone, additional virulence chaperones exist for the SPI-2 encoded T3SS and its effectors, and that SsaN is involved in effector secretion at the cytoplasmic face of the T3SS has been a guiding theme throughout my graduate studies.

The above goals have been achieved and are a significant contribution to the field of *S. enterica* pathogenesis. Two virulence chaperones have been identified that are associated with the SPI-2 encoded T3SS. These proteins are necessary for full virulence and identify the secretion process for two highly studied and critical effectors, and an essential protein of the translocon system. In addition, functional characterization of the T3SS ATPase SsaN has commenced and the work described here will be the foundation for identifying additional functional motifs in this enzyme. Taken together these studies increase our understanding the of SPI-2 mediated intracellular survival of *S. enterica*.

Figure 1.1 – Schematic of the SPI-2 encoded T3SS. (A) The proteins encoded within SPI-2 and their localization based on localization studies with homologous T3SSs are depicted. The sorting platform is likely associated with the T3SS in acidic pH conditions to block effector secretion and allow translocon secretion, it is shown dissociated here. The virulence chaperone SscB is bound to the T3SS with its effector substrate SseF. SseF is also shown secreted into the host cell cytoplasm (IM-inner membrane, OM-outer membrane, HCM-host cell membrane). (B) The genes encoded within SPI-2 are listed in their respective operons, shaded according to known or predicted function. Promoters are shown at the beginning of operons with arrows (R-regulatory, A-apparatus, E/C-effector chaperone).

Figure 1.1

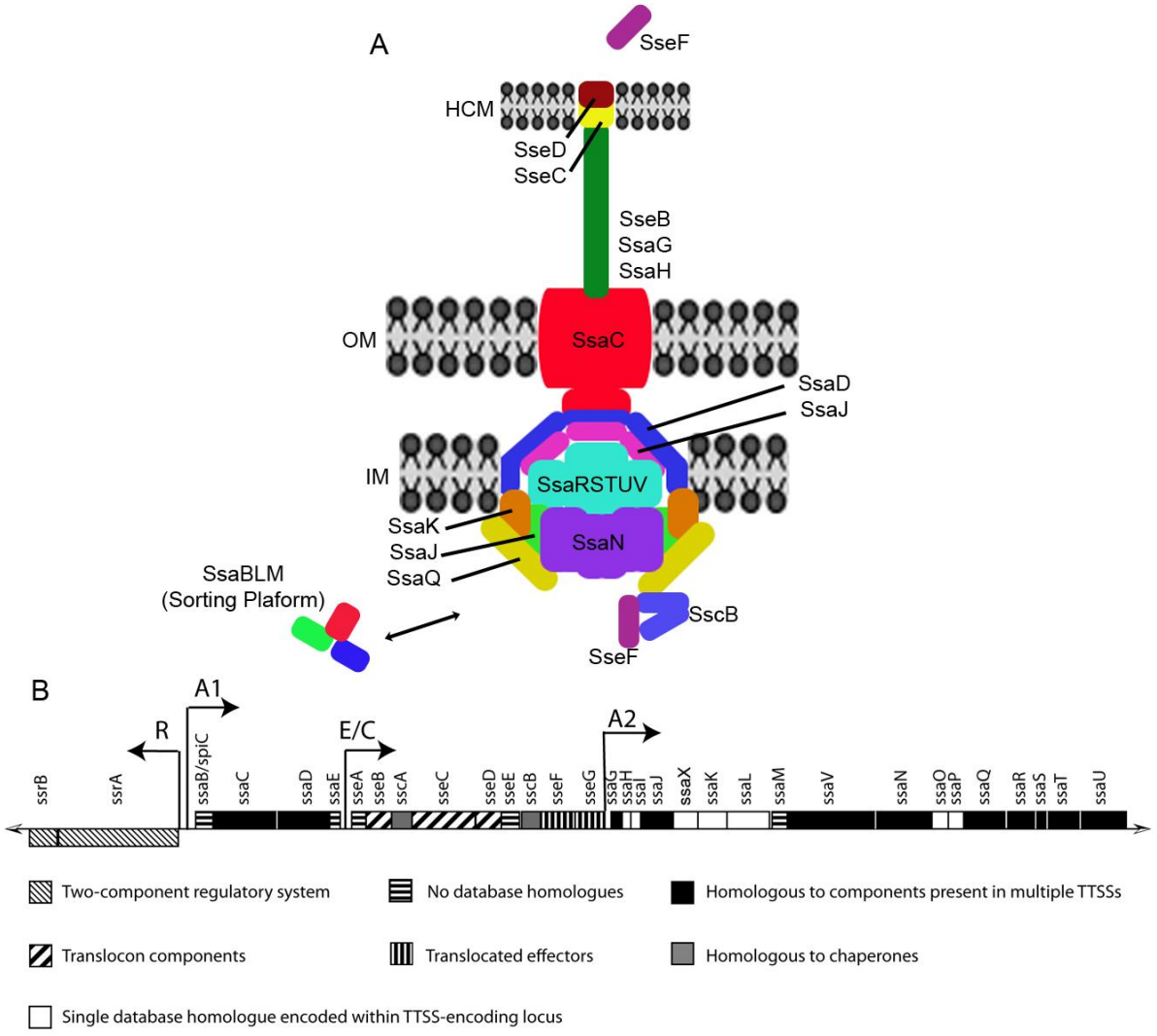

A
SseF
HCM
SseD
SseC
SseB
SsaG
SsaH
OM
SsaC
SsaD
SsaJ
IM
SsaRSTUV
SsaK
SsaJ
SsaQ
SsaN
SsaBLM
(Sorting Plaform)
SscB
SseF
B
A1
R
E/C
A2
ssrB
ssrA
ssaB/spiC
ssaC
ssaD
ssaE
sseA
sseB
sscA
sseC
sseD
sseE
sscB
sseF
sseG
ssaG
ssaH
ssaI
ssaJ
ssaX
ssaK
ssaL
ssaM
ssaV
ssaN
ssaO
ssaP
ssaQ
ssaR
ssaS
ssaT
ssaU
Two-component regulatory system
No database homologues
Homologous to components present in multiple TTSSs
Translocon components
Translocated effectors
Homologous to chaperones
Single database homologue encoded within TTSS-encoding locus

Figure 1.2 – Translocon platform types in *Yersinia* and EPEC. (A) The needle filament YscF, the single, punctuate sorting platform LcrV, and the two translocon components YopB and YopD are depicted. (B) The needle filament EscF is shown localized with the filamentous translocon protein EspA, creating a sheath-like needle filament. The translocon components EspB and EspD are supported by EspA (HCM-host cell membrane).

Figure 1.2

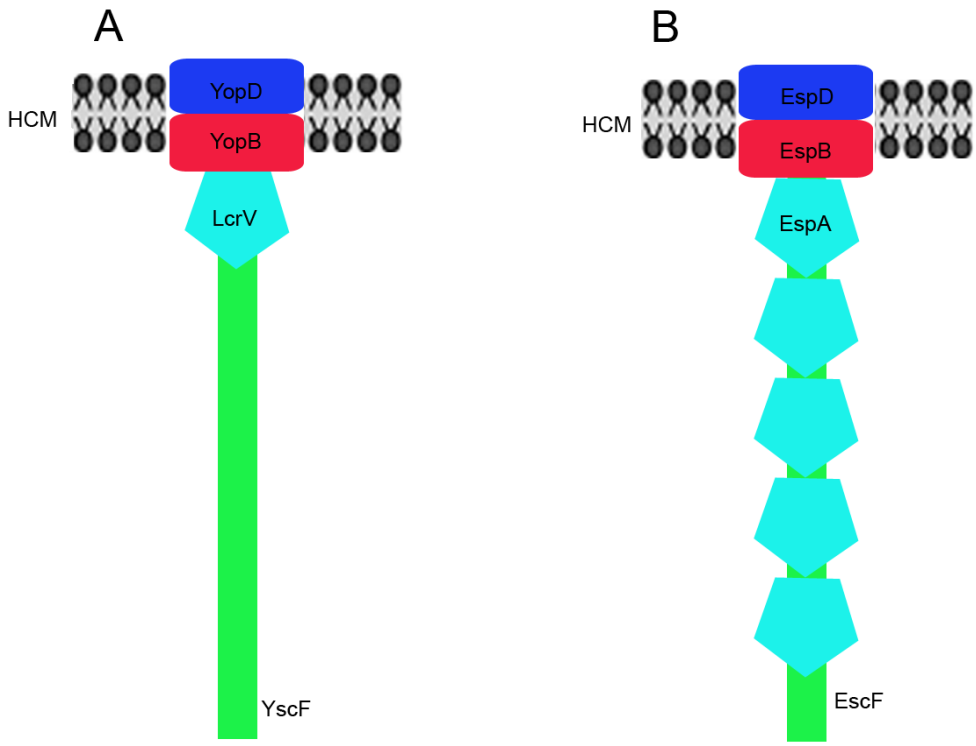

A
HCM
YopD
YopB
LcrV
YscF
Single translocon platfrom
(*Yersinia*)
B
HCM
EspD
EspB
EspA
EscF
Filamentous translocon platform
(EPEC)

Figure 1.3 – Structural representation of class II virulence chaperones. Crystal structure of IpgC (*Shigella*) in complex with a peptide from the translocon protein IpaD (A) (PDB 3GZ1). One monomer is labelled in green, the other is red, while the IpaD peptide is blue. Structural representation of SycD (*Yersinia*) (B) (PDB 2VGX), one monomer is labelled cyan, the other yellow. Note the concave substrate binding face similar in each chaperone, shown with the green monomer of IpgC, and the cyan monomer of SycD. When modelled to highlight these motifs, it is clear that a conserved structural motif is used for substrate binding, but not necessarily for dimer interactions.

Figure 1.3

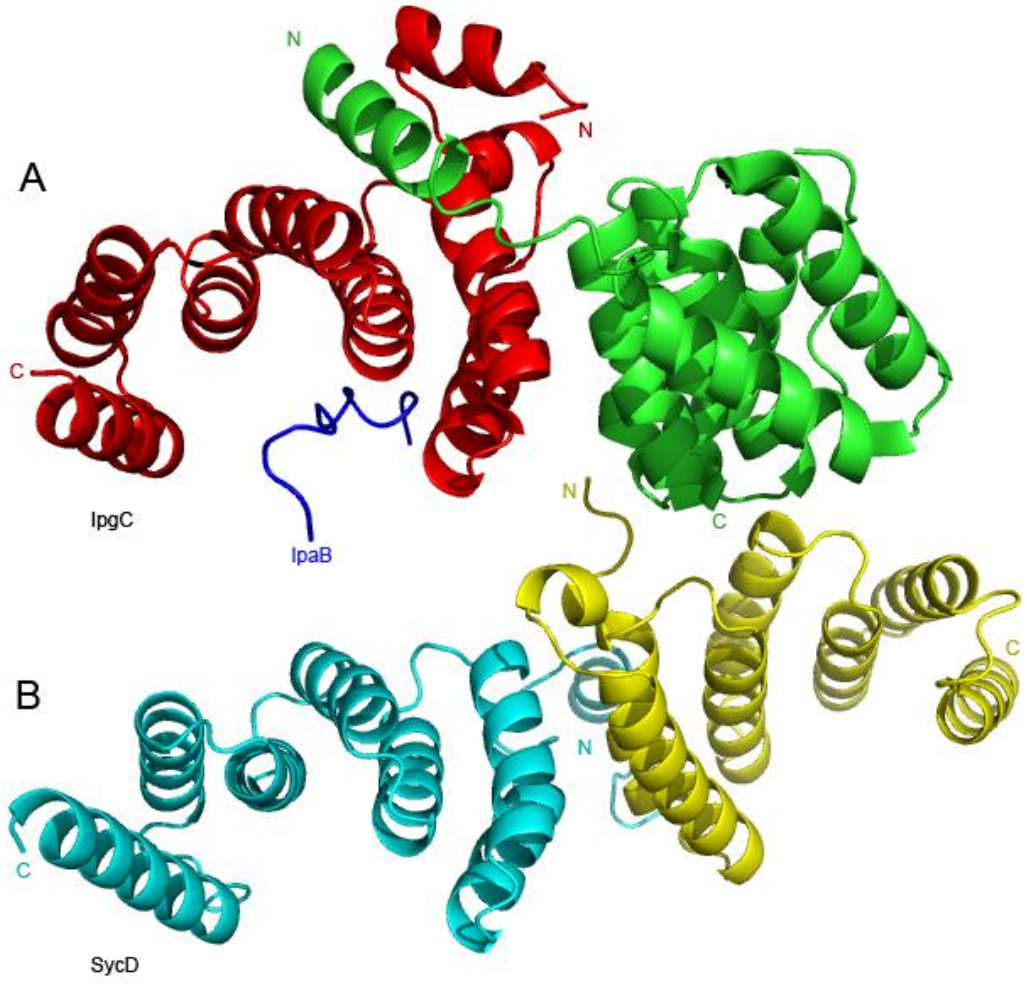
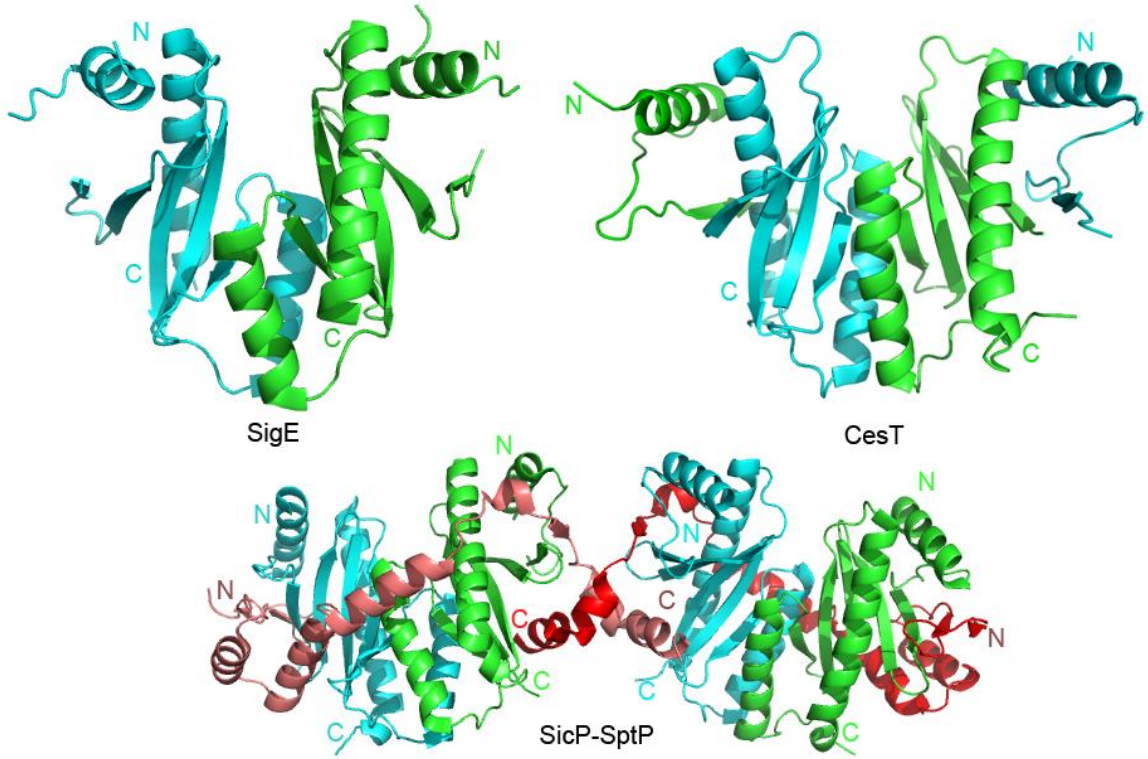


Figure 1.4 – Structural representations of class I virulence chaperones. Shown are SigE (top left) (PDB IK3S), CesT (top right) (PDB IK3E), and SicP bound to peptides (residues 35-139) of its effector substrate SptP (bottom) (PDB 1JYO). All proteins display the typical horseshoe-like structure although CesT uses a domain swap for dimerization in addition to the central α-helix interaction site. SicP is shown in the predicted biologically relevant dimer of dimers conformation where two horseshoe-like dimeric structures are connected by the SptP effector cargo. One protein monomer is labelled in green, and the other in cyan, while the SptP effector peptides are labelled in red or pink.

Figure 1.4



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Chapter II - Chapter Two - Structural and biochemical characterization of SrcA, a multi-cargo type III secretion chaperone in Salmonella required for pathogenic association with a host

Chapter II – Co-authorship statements

Chapter II is composed of the following publication:

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2) Competitive Infection data was obtained by BKC.

3) Protein purification, gel filtration experiments, and crystal generation were performed by KZ.

4) Diffractions, data collection, and refinement of the SrcA crystal structure was performed by MSJ and SNA. Crystal structure figures were constructed by MSJ and SNA.

5) Mass spectrometry was carried out by YF, and analyzed by YF and LJF.

6) SCV localization experiments were carried out by NAK, and analyzed by NAK and JHB.

7) The final manuscript was edited by JHB, LJF, MSJ, and BKC.

Structural and biochemical characterization of SrcA, a multi-cargo type III secretion chaperone in *Salmonella* required for pathogenic association with a host

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Abstract

Many Gram-negative bacteria colonize and exploit host niches using a protein apparatus called a type III secretion system (T3SS) that translocates bacterial effector proteins into host cells where their functions are essential for pathogenesis. A suite of T3SS-associated chaperone proteins bind cargo in the bacterial cytosol, establishing protein interaction networks needed for effector translocation into host cells. In *Salmonella enterica* serovar Typhimurium, a T3SS encoded in a large genomic island (SPI-2) is required for intracellular infection but the chaperone complement required for effector translocation by this system is not known. Using a reverse genetics approach, we identified a multi-cargo secretion chaperone that is functionally integrated with the SPI-2-encoded T3SS and required for systemic infection in mice. Crystallographic analysis of SrcA at a resolution of 2.5 Å revealed a dimer similar to the CesT chaperone from enteropathogenic *E. coli* but lacking a 17-amino acid extension at the carboxyl terminus. Further biochemical and quantitative proteomics data revealed three protein interactions with SrcA, including two effector cargos (SseL and PipB2) and the type III-associated ATPase, SsaN that increases the efficiency of effector translocation. Using competitive infections in mice we show that SrcA increases bacterial fitness during host infection, highlighting the *in vivo* importance of effector chaperones for the SPI-2 T3SS.

Author Summary

Systemic typhoid fever caused by *Salmonella enterica* serovar Typhi leads to high mortality in the developing world and can be linked with chronic, persistent infections in survivors. To cause disease, *Salmonella* uses a specialized secretion device called a type III secretion system to disarm cells of the immune system and replicate within them. The assembly and function of this secretion system requires a set of chaperone proteins to direct the process, but the chaperone proteins themselves have remained elusive. Here, we found a new chaperone protein, called SrcA, which is required for proper function of the type III secretion system. Using a bacterial mutant lacking the *srcA* gene, we found that this chaperone was needed for *Salmonella* to compete against wild type cells during systemic disease because it controls secretion of at least 2 key proteins involved in immune escape and cell-to-cell transmission. This chaperone is present in all types of virulent *Salmonella*, but not in *Salmonella* that don't cause human infections, providing new insights into the pathogenic nature of this organism.

Introduction

Many Gram-negative bacteria that colonize host animals use a type III secretion system (T3SS) to deliver effector proteins directly into host cells where their interaction with host proteins and membranes contribute to pathogenesis. Comprised of over 20 proteins, T3SS are complex structures with relation to the flagellar T3SS [1,2] and include several central features; (i) inner and outer membrane ring structures, (ii) an extracellular needle structure with pore-forming proteins at the distal tip that engage a host cell membrane, (iii) an ATPase at the base of the apparatus with energetic and chaperone-effector recruitment roles, and (iv) a suite of chaperones to coordinate the assembly and function of the apparatus during infection.

Secretion chaperones are proteins required for T3SS function with roles in apparatus assembly and effector delivery, but are not themselves subject to secretion [3]. These chaperones often have common physical features such as low molecular weight (<15 kDa), an acidic isoelectric point and a predicted amphipathic helix at the carboxyl terminus. Current literature groups secretion chaperones into three classes based on their physical interactions with cargo [3,4]. Class I chaperones bind to translocated effector proteins at a chaperone binding domain (CBD) located in the amino terminus of the effector. Class I chaperones have a structural fold of five β-strands and three α-helices, forming homodimers that bind to the CBD in a horseshoe-like structure. These chaperones have been further sub-classified based on their substrate repertoire and location with respect to the genes encoding the T3SS [3]. Class II chaperones bind to translocon proteins that make up the secretion pore in the host target membrane and class III chaperones bind the extracellular filament proteins (or flagellin rod in the orthologous flagellar system) that polymerize into a helical structure following secretion from the bacterial cell.

Secondary structure predictions suggest class III chaperones adopt an extended alpha helical structure, which was confirmed by the crystal structure of the CesA chaperone in enteropathogenic *E. coli* that binds the EspA filament protein [5].

Much of the virulence potential of *Salmonella enterica*, a group of more than 2300 serotypes, is attributed to horizontally acquired genomic islands termed *Salmonella* Pathogenicity Islands (SPI). SPI-1 encodes a T3SS required for host cell invasion and SPI-2 encodes a second T3SS needed for intracellular survival and immune evasion [6,7]. To date, 13 effectors have been identified as substrates of the SPI-1 T3SS and 21 effectors for the SPI-2 T3SS, although the chaperones orchestrating the latter system have been elusive. Whereas 80% of the effectors of the SPI-1 system have defined chaperones, only two effector-chaperone interactions are known for the SPI-2 system. These include the effector-chaperone pair of SseF-SscB, and the chaperone SseA that binds translocon components SseD and SseB [8,9,10]. Crystal structures have been determined for three chaperones that coordinate translocation of effectors through the SPI-1 T3SS (InvB [11], SicP [12] and SigE [13]). However no structures are available for the SPI-2 T3SS chaperones whose effector repertoire seems considerably larger than that of the SPI-1 system.

In addition to maintaining a region of localized effector unfolding [12], T3SS chaperones have an emerging role as escorts that deliver their cargo to the cytoplasmic face of the inner membrane through physical interactions with an ATPase. These ATPases form a hexameric structure at the base of the T3SS [14] and are a conserved feature of both flagellar and non-flagellar type III systems to enhance secretion activity by promoting chaperone release and effector unfolding prior to secretion [15,16,17]. A chaperone-ATPase interaction for the SPI-2

T3SS has not been described previously and so whether this system conforms to the emerging escort paradigm is not known.

The regulation of the SPI-2 T3SS and its associated effector genes is coordinated by environmental cues signifying the intracellular environment [18]. These cues activate a two-component signaling system encoded in the SPI-2 island comprising the SsrA sensor kinase and SsrB response regulator. In addition to activating all of the T3SS structural operons, transcriptional profiling has uncovered new genes in the SsrB regulon that are required for bacterial pathogenesis including a translocated effector, SseL [19,20], and a gene of unknown function called *srfN* that is common to the *Salmonella* genus [21]. Using a reverse genetics approach we identified an SsrB-regulated gene (STM2138) that we named *srcA* (SsrB-regulated chaperone A), whose gene product satisfied several a priori predictions relating to the physical properties associated with T3SS chaperones. We solved the crystal structure of SrcA and performed additional biochemical, proteomic and *in vivo* experiments that revealed SrcA to be a class I chaperone required for bacterial fitness in the host environment. Despite being genetically disconnected from SPI-2, SrcA is integrated functionally with this system by binding to the T3SS ATPase, SsaN, and providing chaperone activity towards two important effectors, SseL (*STM2287*) and PipB2 (*STM2780*), necessary for immune escape and cell-to-cell transmission. These data reveal structural and biochemical insight into a T3SS secretion chaperone required for intracellular pathogenesis of *Salmonella*.

Results

Identification of an SsrB-regulated secretion chaperone

Transcriptional profiling of SsrB-regulated genes in *S. enterica* serovar Typhimurium (*S.* Typhimurium) [22] identified a hypothetical gene, *STM2138* (named *srcA* hereafter), that was co-regulated with genes in SPI-2 and repressed ~20-fold in an *ssrB* mutant compared to wild type. This gene was also down regulated in *Salmonella* mutants lacking the SsrA sensor kinase [20], and was predicted to encode a possible chaperone in a bioinformatics-based screen [23]. The *srcA* gene is not located in the vicinity of the T3SS encoded by SPI-2 (*STM1378-STM1425*), but is 713 genes downstream on the chromosome (STM numbers are based on the LT2 genome and ordered sequentially on the chromosome beginning at STM0001, *thrL*). The predicted *srcA* *g*ene product was a small protein ~16 kDa with a pI of 4.6, similar to secretion chaperones associated with T3SS. To verify SsrB input on *srcA* expression, we analyzed SsrB binding *in vivo* at the region of DNA surrounding *srcA* using genome-wide ChIP-on-chip [21] (and unpublished data). This analysis revealed a strong SsrB binding site spanning 10 syntenic probes within the intergenic region (IGR) upstream of *srcA*, that together with the transcriptional data corroborated a direct regulatory role for SsrB on *srcA* expression (**Fig. 2.1a**). To determine the cellular distribution of SrcA we constructed a *srcA-HA* allele and expressed this gene in wild type and in *ssrB* mutant cells under conditions that activate the SPI-2 T3SS [24]. In whole cell lysates, SrcA protein was reduced ~10-fold in Δ*ssrB* cells compared to wild type (**Fig. 2.1b**) and the protein was not detected in the secreted fraction from wild type cells (**Fig. 2.1c**), consistent with the expected properties of a T3SS chaperone. As a positive control, SseC, an SsrB-regulated translocon protein of the SPI-2 T3SS was present in the secreted fraction from wild type cells but not from an *ssrB* mutant.

SrcA contributes to *Salmonella* fitness in an animal host

Most SsrB-regulated gene products contribute to the intracellular survival of *Salmonella* in a host. In comparative genomics analyses, *srcA* was found in all virulent strains of *Salmonella enterica* containing SPI-2, but was absent from the cold-blooded animal commensal, *S. bongori*, which lacks SPI-2 (**Table 2.S1**). This suggested a co-evolution of *srcA* with the SPI-2 T3SS and a possible functional relationship. If so, we reasoned that SrcA should contribute to animal colonization because the SPI-2 T3SS is essential for host infection. To determine whether SrcA contributes to *Salmonella* fitness in a host, we created an unmarked in-frame *srcA* deletion in *S.* Typhimurium and competed this strain against wild type cells in mixed oral infections of mice [25]. After three days of infection the geometric mean competitive index (CI) for the mutant was 0.20 (95%CI 0.13-0.29) and 0.18 (95%CI 0.06-0.5) in the spleen and liver respectively (P<0.0001; **Fig. 2.1d**) indicating that bacteria lacking *srcA* were significantly out competed by wild type cells during systemic infection. To verify the role of *srcA* on this phenotype, we complemented the *srcA* mutant with a wild type *srcA* gene under the control of its endogenous promoter, which restored *in vivo* fitness to that of wild type (**Fig. 2.1d**). The level of attenuation of the *srcA* mutant was generally higher than most single effector gene mutants [26], which suggested to us that SrcA contributes to an important aspect of T3SS function *in vivo*.

Crystal structure of SrcA

Sequence analysis showed 59% amino acid identity between SrcA and CesT, a secretion chaperone in enteropathogenic *E. coli* (EPEC) (**Fig. 2.2a**). As a means to address the biological function of SrcA, we solved the crystal structure at 2.5 Å resolution (**PDB 3EPU**). A summary of crystallographic data collection and model refinement statistics is in **Table 2.1**. The structure

was solved by molecular replacement using an initial model based on CesT (PDB 1K3E) [13]. SrcA crystallized in space group C2 with two molecules related by a 2-fold symmetry axis in each asymmetric unit (**Fig. 2.2b**). Each monomer consisted of a small and large domain. The smaller domain formed by α1 and the extended loop region preceding β1 adopts a distinct conformation in each subunit. The larger domain mediates dimerization and is comprised of a twisted anti-parallel β-sheet (β1-β2-β3-β5-β4) flanked by α-helices α2 and α3. The dimer interface formed between SrcA monomers occurs primarily through reciprocal hydrophobic interactions between α2 and α2' with additional interface-stabilizing interactions occurring between the α2 helix of one monomer and β4 and β5 strands of the opposing monomer (**Fig. 2.2b**). The total surface area buried at the dimer interface is 1258 $Å^2$, suggesting that SrcA would exist as a dimer in solution, which we confirmed by gel filtration analysis (see below).

A structure similarity search with SrcA revealed proteins identified as T3SS secretion chaperones. CesT and SicP were the most structurally similar to SrcA, aligning with RMSD of 1.8 Å and 2.2 Å respectively. With the exception of CesT, SrcA has weak overall sequence identity (< 20%) with other T3SS chaperones. CesT, SicP and SrcA contain several clusters of highly conserved amino acids notable on primary sequence alignments (**Fig. 2.2a**). Most of these conserved sites are located in the α2-interface helix and in strands β4 and β5 that help stabilize this interface. Although the N-terminus of these proteins is conserved structurally, the tertiary structures differ for each protein. In CesT, α1 and β1 adopt an extended conformation while the equivalent domain in SicP remains closely packed against the dimerization domain [12]. In SrcA, both extended and closely packed conformations are observed in separate subunits of the same dimer within the asymmetric unit. In the extended conformation the N-terminal helix from one dimer interacts with the β4 region of an adjacent dimer, similar to a domain swap seen in

CesT [13]. At this time, the possible biological relevance for such a domain swap is unclear and may reflect an artifact of crystallization as critically discussed [13].

A comparison of the SrcA dimer interface with other class I chaperone family members indicates the overall similarity of quaternary structure shared between SrcA, CesT and SicP **(Fig. 2.3a)**. This is in contrast to the class II chaperone interface of Spa15, which despite having similar tertiary structure to SrcA adopts a distinct dimer interface. A structural alignment of SrcA and Spa15 generated through alignment of single monomers shows the relative difference in subunit orientation between SrcA and Spa15 reflected by the positions of each monomer in the dimer configuration. These unique orientations produce an 80° rotational offset between respective subunits and could be expected to influence the mode of effector interactions utilized by these proteins.

To evaluate the potential for an effector-binding surface on SrcA, the structure of SicP in complex with its effector SptP was aligned with SrcA and represented as a space-filling model (**Fig. 2.3b**). Binding of SptP occurs primarily in the N-terminus of SicP [12], which is similar to the effector binding surface for SrcA predicted *in silico*. This surface contains several conserved hydrophobic residues including L16, D24, N26, and I32 **(Fig. 2.2a),** which is consistent with SrcA using a similar mechanism for interaction with effectors.

SrcA interacts with the SPI-2 T3SS ATPase

An emerging function for T3SS chaperones is delivery of cargo to the base of the apparatus through interactions with an ATPase. This was shown for the flagellar T3SS [17] and later in the virulence-associated T3SS in *E. coli* [16,27] and the SPI-1 T3SS in *Salmonella* [15]. However, analogous interactions have not been described for the SPI-2 T3SS. Since *srcA* expression was co-regulated with genes in SPI-2, we hypothesized that it had a functional role in

this system. To address this biochemically we purified SrcA and the predicted ATPase for the SPI-2 T3SS, SsaN, and performed binding experiments and gel filtration chromatography of the protein mixtures. SsaN contains conserved amino acid residues characteristic of Walker-A and Walker-B motifs of P-loop nucleoside triphosphate hydrolases, as well as a number of residues shown to contribute to ATP binding or ring stacking with the adenine base of ATP in the *E. coli* orthologue, EscN, (Q412, E191, R366) (**Fig. 2.S1**). Since SsaN had not been characterized biochemically we first verified that our purified protein had ATPase activity (**Fig. 2.S1**). We then mixed SrcA and SsaN proteins and resolved the protein complexes by gel filtration chromatography. By itself, SrcA existed as a dimer in solution (**Fig. 2.4a**) with no higher oligomers present, substantiating the stoichiometry obtained from our crystal data. SsaN existed as a monomer with a minor population eluting in a volume consistent with a probable dimer (**Fig. 2.4b**). When SrcA was mixed with SsaN, a new protein complex of high molecular weight was observed, along with diminished peaks corresponding to the SrcA dimer and SsaN monomer (**Fig. 2.4c**). This new complex elutes with a Stokes radius consistent with an apparent molecular mass of ~600 kDa. We verified the identities of protein originating from each peak by western blot (**Fig. 2.4d**) and LC-MS/MS, which showed the new complex was comprised of both SsaN and SrcA.

SrcA binds effector cargo destined for the SPI-2 T3SS

Since structural and biochemical data unambiguously defined SrcA as a T3SS-associated chaperone, we used two experimental approaches to identify SrcA cargo(s). First, we used stable isotope labeling of amino acids in cell culture (SILAC) [28] in conjunction with quantitative mass spectrometry-based proteomics to identify cargo immunoprecipitated with SrcA from

Salmonella. For this series of experiments we constructed a mutant in which the *srcA* gene was replaced on the chromosome with *srcA-FLAG* to enable immunoprecipitation from cell lysates. Lysates prepared from wild type cells grown in $^{2}H_{4}$-Lys and $^{13}C_{6}$-Arg containing SILAC medium (heavy) and *srcA* mutant cells grown in medium containing natural amino acids of Lys and Arg (light) were mixed and subjected to an immunoprecipitation procedure with an anti-FLAG antibody followed by quantitative mass spectrometry. Peptides originating from wild type cells contained heavy atom-substituted lysine and arginine such that putative SrcA cargo proteins would generate low heavy:light SILAC peptide ratios from the complex mixtures (**Fig. 2.5a**). In these experiments the T3SS effector protein SseL was identified by quantitative SILAC mass spectrometry as a specific SrcA cargo protein **(Fig. 2.5b)**. SseL was immunoprecipitated specifically along with SrcA-FLAG with a SILAC ratio of 0.08, whereas additional abundant proteins displayed SILAC ratios closer to ~1 (OmpF is shown, **Fig. 2.5b)** (mean SILAC ratio of all other peptides identified was 0.93).

Secondly, to verify the mass spectrometry data and to identify other possible effector cargo, we examined the secretion profiles of wild type cells and an *srcA* mutant that each expressed HA-tagged effector genes, the products of which are secreted by the SPI-2-encoded T3SS. Using this approach SseL-HA and PipB2-HA were depleted from the secreted protein fraction of *srcA* mutant cells **(Fig. 2.5c)** but reached similar levels in the bacterial cytoplasm **(Fig. 2.5d)**. As expected from data with the complemented mutant *in vivo*, expression of *srcA* in trans restored effector secretion in the *srcA* mutant (data not shown).

SrcA is required for PipB2-dependent centrifugal displacement of the *Salmonella*-containing vacuole

To further show a role for SrcA in chaperoning PipB2, we set up experiments to test whether deleting *srcA* would phenocopy Δ*pipB2* cells for PipB2-dependent centrifugal displacement of the *Salmonella* containing vacuole (SCV) in epithelial cells, an event linked to cell-to-cell transfer during infection *in vitro*[29]. At 10 h after infection the majority of SCVs were situated near the nucleus in accordance with previous work (**Fig. 2.6a**) [29]. By 24 h after infection SCVs containing wild type bacteria were displaced centrifugally towards the cell periphery whereas SCVs containing either *pipB2* or *srcA* mutant bacteria remained juxtaposed to the nucleus (**Fig. 2.6a**). The average distance from the nucleus of LAMP1+ SCVs containing wild type bacteria was 2.19 μm at 10h post infection and increased to 7.86 μm by 24 h after infection. Conversely, SCVs containing either Δ*pipB2* cells or Δ*srcA* cells were 1.38 μm and 2.09 μm at 10h but lacked centrifugal displacement at 24 h (2.23 μm and 2.85 μm, respectively) (**Fig. 2.6b**).

Discussion

Structural features of SrcA

We used a reverse genetics approach to define a new secretion chaperone in *S.* Typhimurium that is integrated functionally with the T3SS encoded by SPI-2, a system well described for its role in immune subversion and intracellular infection during host colonization. Consistent with other class I secretion chaperones, SrcA has extensive electronegative charge distributed over the surface of the molecule. The exact function of this charge distribution is not known, but data from other systems suggests a docking recognition function with other

components of the type III apparatus, possibly the T3SS-accociated ATPase. For instance, electronegative surface residues on the SigE chaperone in the SPI-1-encoded T3SS negatively affect cargo secretion, but not cargo stability [30]. In enteropathogenic *E. coli*, a surface-exposed electronegative residue in the CesT chaperone (Glu142) likewise contributes to Tir secretion but not Tir binding [16], suggesting a role in either targeting bound cargo to the T3SS or in the secretion process itself. Interestingly, SrcA lacks 17-amino acids that make up the carboxyl terminus of CesT, which includes Glu142, and yet it still retains effector binding, ATPase binding and effector secretion functionalities. Thus, it is likely that other surface charged residues of SrcA are involved in these functions or that SrcA targets effector cargo to the secretion apparatus through a mechanism distinct from CesT.

The interface for the SrcA homodimer is extensive and is more in keeping with the structural features of single-effector class IA chaperones (~1100-1300 $Å^2$) compared to the reduced dimer interface of Spa15, a multi-cargo class IB chaperone from *Shigella* [31]. Similar to CesT and SicP, the dimer interface of SrcA adopts a parallel configuration when comparing $\alpha 2$ helices of opposing subunits. In contrast, the subunits of Spa15 undergo a significant relative rotation (80°) about the $\alpha 2$-axis resulting in a different interface. These features may relate to biological function in the SPI2 T3SS and/or in vetting effector cargo amongst the >30 effectors identified in *Salmonella*. We found no evidence of interactions between SrcA and translocon components of the SPI-2 T3SS and so it appears as though SrcA functions specifically in effector translocation events.

Implications for type III secretion function

The interaction between SrcA and SsaN supports an emerging paradigm whereby secretion chaperones bring effector cargo to the T3SS through physical interaction with the hexameric ATPase at the base of the apparatus [14]. This was demonstrated for chaperone-ATPase components in the flagellar type III system [17] and in non-flagellar type III systems in *E. coli* [27] and the SPI-1 system in *Salmonella* [15]. Our work shows the first chaperone-ATPase interaction for a T3SS functioning from within an intracellular vacuolar compartment and supports this interaction as a more generalized feature of type III secretion function. In our experiments, we could induce the ATPase domain of SsaN to oligomerize in the presence of SrcA, but not in its absence, which was intriguing because the purified enzyme lacked a domain at the carboxyl terminus thought to be involved in oligomer stability, at least for *E. coli* EscN [14]. These data suggest that type III chaperones might have an as yet undefined role in assembly of the ATPase homohexamer that gives rise to efficient effector translocation. This will be an important area for further experimentation in this and other systems.

Genetic and functional integration of SrcA with type III secretion

The genes encoding the *srcA* chaperone and the effector cargos (*pipB2* and *sseL*) are found in all serotypes of *Salmonella enterica* that contain the SPI-2-encoded T3SS. Conversely, these genes are absent from *S. bongori*, which lacks the SPI-2-encoded T3SS. The expression of *srcA* is coordinated with T3SS transcriptional activity via the SsrA-SsrB two-component regulatory system encoded in SPI-2. The direct binding of SsrB to the promoter region upstream of *srcA*, along with SsrB-regulation of both *sseL* [19,20] and *pipB2* [32] is indicative of multiple *cis*-regulatory mutation events that have allowed for functional coordination of the distributed

secretion apparatus, chaperone and effector cargos. We recently described this type of regulatory evolution for pathogenic adaptation of *Salmonella* to its host [21] and *srcA* is consistent with regulatory evolution of chaperone-effector gene pairs that are not co-transcribed in operons.

Due to low G+C base content compared to the genome average of 52%, it's likely that *srcA* (32% G+C) and an adjacent gene, *STM2137* (37% G+C), were acquired as a foreign islet that was retained in organisms containing the SPI-2 T3SS due to the selective advantage afforded by the new protein interactions so created. Interestingly, STM2137 (also known as SseK2) is a likely paralog of SseK1, an effector translocated by the SPI-2 T3SS [33]. SseK2 is also regulated by the SsrA-SsrB two-component system but compared to SseK1, it is translocated in much less abundance into host cells [33]. Using the methods described here, we were not able to detect SseK2 secretion or a physical interaction with SrcA, however it remains possible that SrcA also chaperones SseK2 for low-level translocation.

SrcA is unique among other multi-effector chaperones most closely related to it in that it is unlinked from the T3SS genomic island. For example, InvB and SicP (*Salmonella* SPI-1), CesT (enteropathogenic *E. coli* locus of enterocyte effacement) and Spa15 (*Shigella mxi/spa* virulence plasmid region) chaperones are all encoded within the T3SS structural operons, implying they have co-evolved as a single genetic entity from a common ancestor. Given its genetic neighborhood, *srcA* appears to be a genetic acquisition separate from SPI-2 that functionally links some effectors to the T3SS apparatus via the ATPase. The role of horizontal gene transfer and regulatory evolution in allowing for uncoupling of chaperones, effectors and the T3SS has many possible implications for T3SS function, including plasticity in chaperone-effector interaction networks, expansion of effector repertoires, and alterations to the kinetics and hierarchical delivery of effectors to a host cell. These events may improve host adaptability or

even expand the host range of bacteria that acquire and integrate new functional secretion chaperones.

Methods

Ethics statement.

All experiments with animals were conducted according to guidelines set by the Canadian Council on Animal Care. The local animal ethics committee, the Animal Review Ethics Board at McMaster University, approved all protocols developed for this work.

Bacterial strains and growth conditions.

Salmonella enterica serovar Typhimurium strain SL1344 was used as the wild type strain and all mutants were isogenic derivatives. Chromosomal replacements were done using a λ-Red-based technique described previously [34]. A synthetic minimal medium for isotopic labeling of proteins in cell culture was developed for SILAC proteomics experiments based on LPM medium that activates the SsrA-SsrB two-component regulatory system for induction of SsrB-regulated genes [24]. LPM medium was modified for compatibility with quantitative SILAC mass spectrometry by replacing casamino acids with individual L-amino acids and containing either natural L-arginine and L-lysine, or ^{13}C-subsituted arginine ($^{13}C_6$-Arg) and deuterium-substituted lysine ($^{2}H_{4,4,5,5}$-Lys) (Cambridge Isotope Laboratories, Andover, MA). A full description of LPM-SILAC medium is provided in Protocol S1.

Protein production and purification.

For purification of His-tagged SrcA, the *srcA* gene was amplified from *S.* Typhimurium chromosomal DNA and cloned into pET-3(a) (Novagen) as a C-terminal fusion to a 6-histidine tag. Expression plasmids were transformed into *E. coli* Rosetta (DE3) and cells were grown in 1-L LB broth and induced with IPTG at OD_{600nm} 0.6 for 3 h at 37°C. Harvested cells were resuspended in 25 ml NiA buffer (20 mM Tris pH 8.5, 500 mM KCl, 20 mM imidazole, 0.03% LDAO and 10% glycerol)**,** lysed using a French press and centrifuged at 48,383 *g* for 40 min. Soluble His-tagged protein was purified using nickel-chelating resin (GE Healthcare Life Sciences), followed by Mono-Q anion exchange using a 20 mM Tris pH 7.5, 500 mM KCl, 10% glycerol. Purified protein was exchanged into a final buffer of 20 mM Tris pH 7.5, 100 mM KCl, 10% glycerol and concentrated to ~5 mg/mL. All SrcA purification steps were carried out at room temperature. For purification of His-tagged SsaN, a soluble protein form containing the ATPase domain and C-terminal domain spanning residues Q90-E433 was constructed according to previous work done on *E. coli* EscN [14]). SsaNΔ89 was purified from *E. coli* Rosetta (DE3) cells containing a pET-3(a) plasmid with the *ssaNΔ89* gene. Cells were sub-cultured into 1-L of Terrific Broth (TB) and grown with shaking at 200 rpm at 20°C for 65 h for auto-induction. Cells were harvested and lysed using a French press and soluble protein was purified using nickel chromatography and ion-exchange chromatography as described above. Finally, SsaN protein was concentrated to ~9 mg/ml. All purification steps for SsaN were carried out at 4°C.

Crystallization, data collection and structure determination.

Crystals were generated via the hanging drop method by vapor diffusion using purified protein combined with crystallization solution (100 mM Bis-Tris propane pH 7.0, 200 mM $MgCl_2$, 35% PEG 3350, 3.95 mM FOS-choline-9, 5% Jeffamine M-600) at a 3.5:1 ratio, and equilibrated over 500 μL of 1.7 M ammonium sulfate, at 298K. After initial crystals were formed, drops were moved over wells containing 500 μL of 3 M ammonium sulfate and further equilibrated for 2 to 3 weeks. A single native data set, collected to 2.5 Å at the National Synchrotron Light Source Beamline X12C (Brookhaven, NY) was processed using HKL2000. An initial structural solution was achieved using molecular replacement with the type III chaperone CesT (PDB ID 1K3E) as the starting search model. PHENIX was used for model building [35]. Further model building and refinement was conducted iteratively using COOT and REFMAC [36,37]. The final structure had R and R_{free} values of 21.4 and 25.3 respectively.

Competitive infection of mice.

For competitive infections, female C57BL/6 mice (Charles River) were infected per os with a 1:1 mixed inoculum containing *srcA* mutant cells and a marked wild type strain resistant to chloramphenicol as described previously [21]. Competitive index (CI) was calculated in the liver and spleen at 3 days after infection as: cfu (mutant/wild type) $_{output}$ / (mutant/wild type) $_{input}$. For complementation experiments, *srcA* was cloned with its native promoter into the low-copy plasmid pWSK29 and transformed into Δ*srcA* cells. The complemented mutant was competed in CI experiments against wild type cells transformed with empty pWSK29.

Co-immunoprecipitation experiments and quantitative SILAC mass spectrometry.

Co-immunoprecipitations were performed with M2-Agarose beads conjugated with anti-FLAG antibody (F-gel, Sigma, Oakville, ON). Wild type bacteria and bacteria with a *srcA-FLAG* allelic replacement were grown overnight in LB broth, washed in SILAC-LPM (Protocol S1), and sub-cultured 1:50 into SILAC-LPM containing either $^{12}C_6$-Arg and H_4-Lys (light) or $^{13}C_6$-Arg and $^{2}H_4$-Lys (heavy) amino acids. Isotopic labeling of proteins was carried out until the culture reached an optical density of 0.6 at 600 nm. Cells were washed with PBS, pelleted at 3000 *g* for 10 minutes and resuspended in PBS containing mini-EDTA tablet (1 per 10 ml) protease inhibitors (PBS-PI) (Roche, Mississauga, ON). Cells were sonicated six times for 30 seconds each with 1 min intervals on ice (Misonix Sonicator 3000, Misonix, Farmingdale, NY). Samples were centrifuged at 3000 *g* for 15 minutes and the resulting supernatants from heavy and light samples were mixed. F-gel was equilibrated with PBS-PI containing 10 µg/ml BSA for 60 minutes and then lysates were immunoprecipitated with F-gel for 16 h at 4°C. F-gel was washed with PBS-PI ten times for 30 min each wash. Bound proteins were eluted with either FLAG peptide or twice with SDS-sample buffer (1 M Tris pH 8.0, 20% SDS, 0.5 M EDTA pH 8, 10% glycerol, 200 mM dithiothreitol). Final protein preparations were filter-concentrated, washed with water and diluted to a final concentration of 50 mM ammonium bicarbonate and 1% sodium deoxycholate. Proteins were digested in solution and analyzed by liquid chromatography-tandem mass spectrometry exactly as described previously [38]

Gel filtration chromatography.

Two hundred microlitres of purified SrcA protein in gel filtration buffer (20 mM Tris pH 7.5, 200 mM KCl; protein concentration, 1.1 mg/ml) was injected into a Superdex S200 10/300GL

gel filtration column (Amersham Biosciences, Piscataway, NJ) at 0.2 ml/min. Elution fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min. For SsaN, 40 µl (8.84 mg/ml) was diluted with 320 µl gel filtration buffer and injected into an S200 column as described. For mixing experiments, 320 µl of SrcA (0.1mg/ml) was mixed with 40.8 µl SsaN (8.84 mg/ml) at room temperature for 2 h. The mixture was centrifuged at 10,000 *g* for 5 minutes and the top two hundred microlitres of the supernatant was injected into an S200 column. Peak fractions were collected and protein identities in all peaks were verified by Western blot and LC-MS/MS.

Type III secretion assays.

Experiments to monitor secretion of type III effectors were performed according to previously published methods [19]. Wild type cells and an *srcA* mutant used for these experiments were transformed with low-copy plasmids expressing HA-tagged effector genes from their endogenous promoters (*sifA, sopD2, gogB, pipB, sseK2*) or contained allelic replacements on the chromosome to express HA-fusion proteins (*pipB2, sseL*). Antibodies used for Western blots were: mouse anti-HA (1:1000), mouse anti-DnaK (1:5000), rabbit anti-SseC (1:20000). Secondary antibodies conjugated to horseradish peroxidase (HRP) were used at 1:5000 and antigen-antibody complexes were detected using enhanced chemiluminescence (ECL).

ATPase assay.

ATPase activity of SsaN was measured using the pyruvate kinase-lactate dehydrogenase coupled assay that monitors NADH oxidation coupled with ATP hydrolysis [39]. Data was plotted as a decrease in absorbance at 340 nm over time.

SCV positioning experiments.

The intracellular position of *Salmonella*-containing vacuoles was determined by measuring the distance of LAMP1+ SCVs to the nearest edge of the host cell nucleus (labeled by DAPI staining) in fixed HeLa cells as previously reported [29]. Measurements were made using Openlab 3.1.7 software. Experiments were done in duplicate and the resulting finalized average was calculated from two independent average values (at least 100 measurements per experiment). Average distances with average deviation are reported.

Coordinates. The coordinates and structure factors of SrcA have been deposited in the Protein Data Bank (accession code 3EPU).

Acknowledgements

We thank members of the Coombes and Junop laboratories for helpful discussions relating to this work.

Author Contributions

Conceived and designed the experiments: CAC KZ NAK MH JHB LJF MSJ BKC. Performed the experiments: CAC KZ YF NAK MH MSJ. Analyzed the data: CAC KZ SNA YF NAK MH JHB LJF MSJ BKC. Contributed reagents/materials/analysis tools: SNA LJF. Wrote the paper: CAC NAK JHB LJF MSJ BKC.

Table 2.1: Crystallographic Data and Refinement Statistics

Data collection	
Wavelength (Å)	1.1
Space group	*C*2
Cell parameters	a=103.84, b=46.83, c=65.94 α=γ=90; β=106.39
Molecules in A.U.	2
Resolution range (Å) [a]	50-2.49 (2.58-2.49)
Unique reflections	10664
Data Redundancy [a]	3.62 (3.58)
Completeness (%) [a]	98.5 (94.6)
$I/\sigma(I)$[a]	13.5 (3.4)
R_{merge}(%)[a]	6.4 (31.6)
Wilson scaling *B* factor (Å^2)	54.8
Model and refinement	
Resolution range (Å)	50-2.5
R_{work} (%)	21.45
R_{free} (%)	25.26
Refl. observed	10170
Refl. test set	538
No. of protein atoms	2174
No. of waters	86
rmsd bond lengths (Å)	0.012
rmsd bond angles (Å)	1.371
Average *B* factor (Å^2)	63.8

[a] Data for the highest resolution shell are shown in parentheses.

Figure 2.1. Identification of an SsrB-regulated gene, *srcA*

(a) Chip-on-chip analysis of the genomic region surrounding *srcA* including the 5' upstream region. *In vivo* binding of SsrB was positive for 10 syntenic probes in the intergenic region (IGR) upstream of *srcA*. Each data point is a unique probe with averaged data from three biological replicates. Direction of gene transcription is shown by arrows beneath the abscissa and includes the fold-change in mRNA levels in *ssrB* mutant cells. **(b)** Accumulation of SrcA protein in cells requires SsrB. Cells harboring an *srcA-HA* allele were grown under SsrB-activating conditions and whole cell lysates (pellet) were probed by western blot for SrcA-HA, which accumulated in wild type cells (wt) but not in *ssrB* mutant cells (Δ*ssrB*). **(c)** SrcA is not secreted into the culture supernatant. Cell-free secreted protein fractions from wt and Δ*srcA* cultures were probed for SrcA-HA. SrcA-HA was not found in the secreted protein fraction from any of the cultures, whereas the type III-secreted protein, SseC, was secreted into the medium in an SsrB-dependent manner. **(d)** SrcA is required for competitive fitness *in vivo*. The ability of *srcA* mutant cells to compete with wild type parent cells was quantified by competitive infections of mice. Mice were infected orally with an equal proportion of mutant and wild type cells and the competitive index in the spleen and liver was determined at three days after infection. Complementation of *srcA* in trans (p*srcA*) restored the ability of *srcA* mutants to compete equally with wild type cells. Each data point represents an individual animal.

Figure 2.1

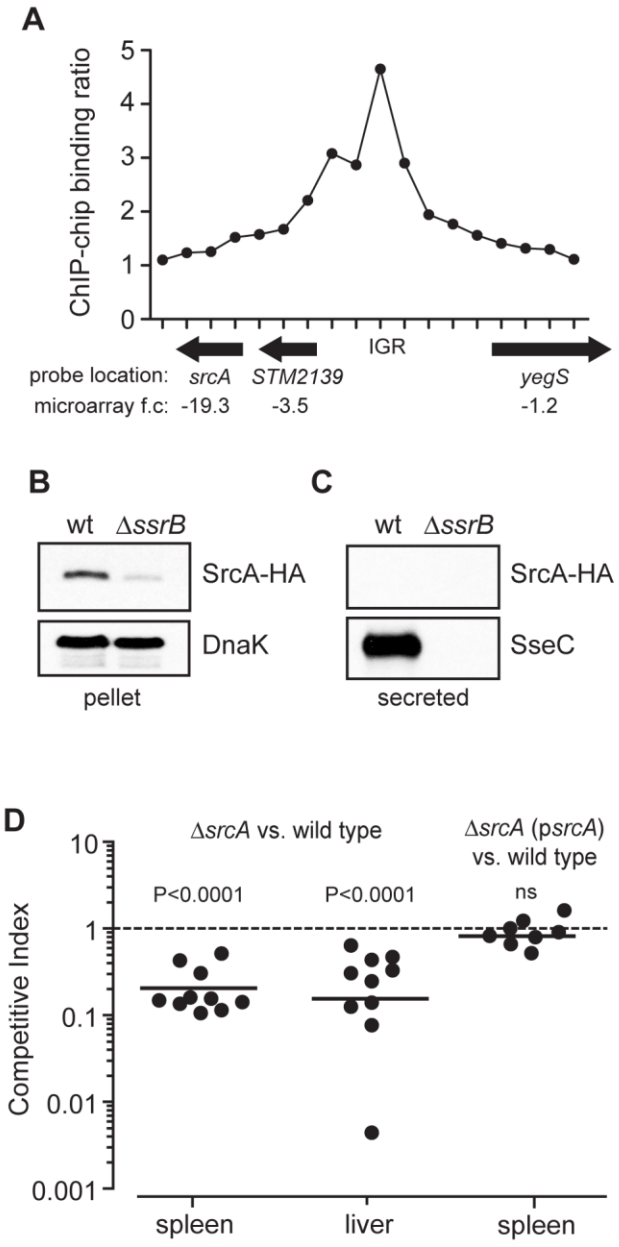

A
ChIP-chip binding ratio
0
1
2
3
4
5
IGR
probe location:
srcA
STM2139
yegS
microarray f.c:
-19.3
-3.5
-1.2
B
wt
ΔssrB
SrcA-HA
DnaK
pellet
C
wt
ΔssrB
SrcA-HA
SseC
secreted
D
ΔsrcA vs. wild type
ΔsrcA (psrcA) vs. wild type
P<0.0001
P<0.0001
ns
Competitive Index
10
1
0.1
0.01
0.001
spleen
liver
spleen

Figure 2.2. Structural characterization of SrcA

(a) Structure-based sequence alignment of full-length SrcA with structural orthologs CesT from enteropathogenic *E. coli* and SicP from *S.* Typhimurium. Orange arrowheads denote residues conserved between all three proteins; black arrowheads denote residues that participate in the cargo-binding interface as defined in [12] (PDB 1JY0). Conserved residues are colored as follows: hydrophobic, yellow; negative charge, red; positive charge, blue; threonine, serine, pink; cysteine, light blue; proline, glycine, green. **(b)** Stereo image of a SrcA dimer with α-helices and β-strands in blue and red respectively.

Figure 2.2

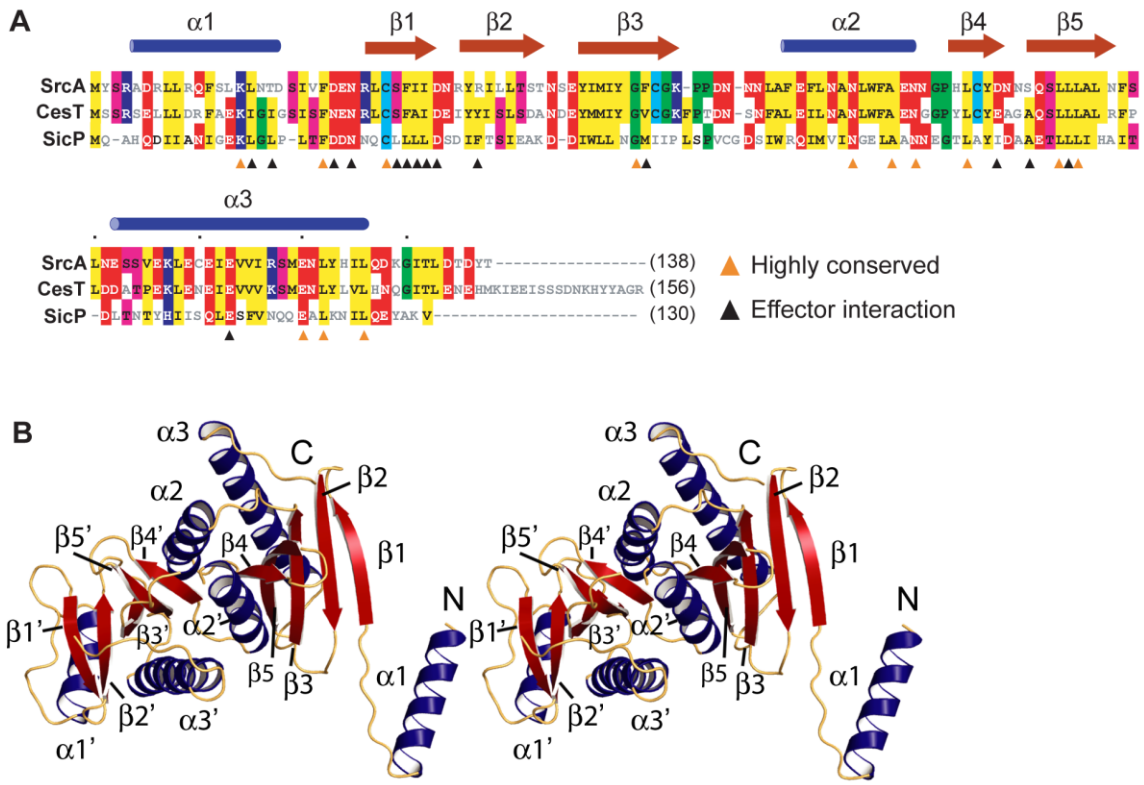

A
α1
β1
β2
β3
α2
β4
β5
SrcA
CesT
SicP
α3
(138)
(156)
(130)
Highly conserved
Effector interaction
B
α3
C
β2
α2
β5'
β4'
β4
β1
β1'
β3'
α2'
β5
β3
N
α1
β2'
α3'
α1'

Figure 2.3. Structural comparison of SrcA, CesT and SicP

(a) Crystal structures of SrcA, CesT, and SicP dimers highlighting the dimer interface. The two monomers of each chaperone dimer are colored in light and dark colors (green, SrcA; yellow, CesT; purple, SicP; orange, Spa15). **(b)** Space-filling model of SrcA bound to the SicP effector, SptP, based on a structural alignment. Negatively charged patches are red, positively charged patches are blue, and hydrophobic patches are white. Black arrows indicate the N-terminus.

Figure 2.3

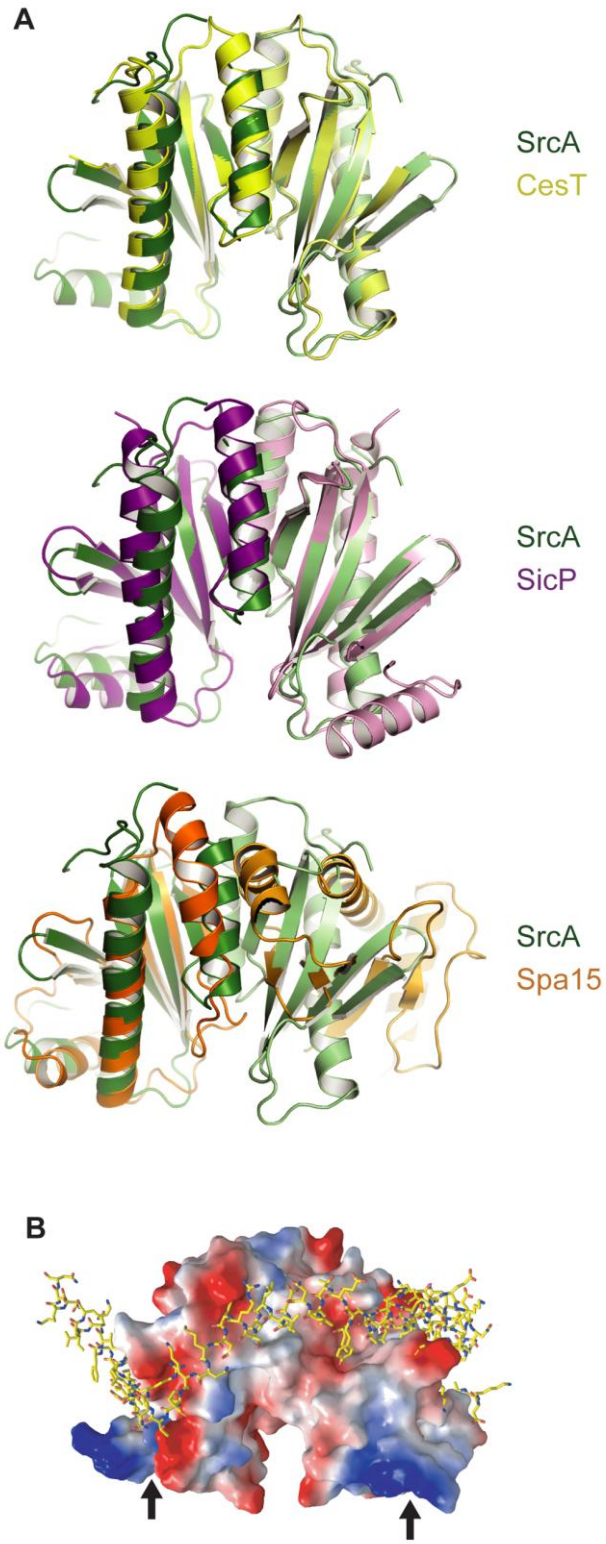


Figure 2.4. SrcA binds to the type III ATPase, SsaN, and induces its multimerization

Gel filtration chromatography was used to analyze **(a)** purified SrcA, **(b)** purified SsaN, and **(c)** a mixture of SrcA with SsaN. **(d)** Peak fractions (black arrows) in the elution volumes were collected and analyzed by western blot with an anti-His antibody to identify protein compositions. Pre-column samples of SrcA migrated as monomer and dimer species in SDS-PAGE gels (verified by LC-MS/MS) while SsaN was prominently a monomer. Incubation of SsaN with SrcA chaperone induced the formation of a high-molecular weight oligomeric species comprised of both proteins, which was only present in SsaN-SrcA mixtures.

Figure 2.4

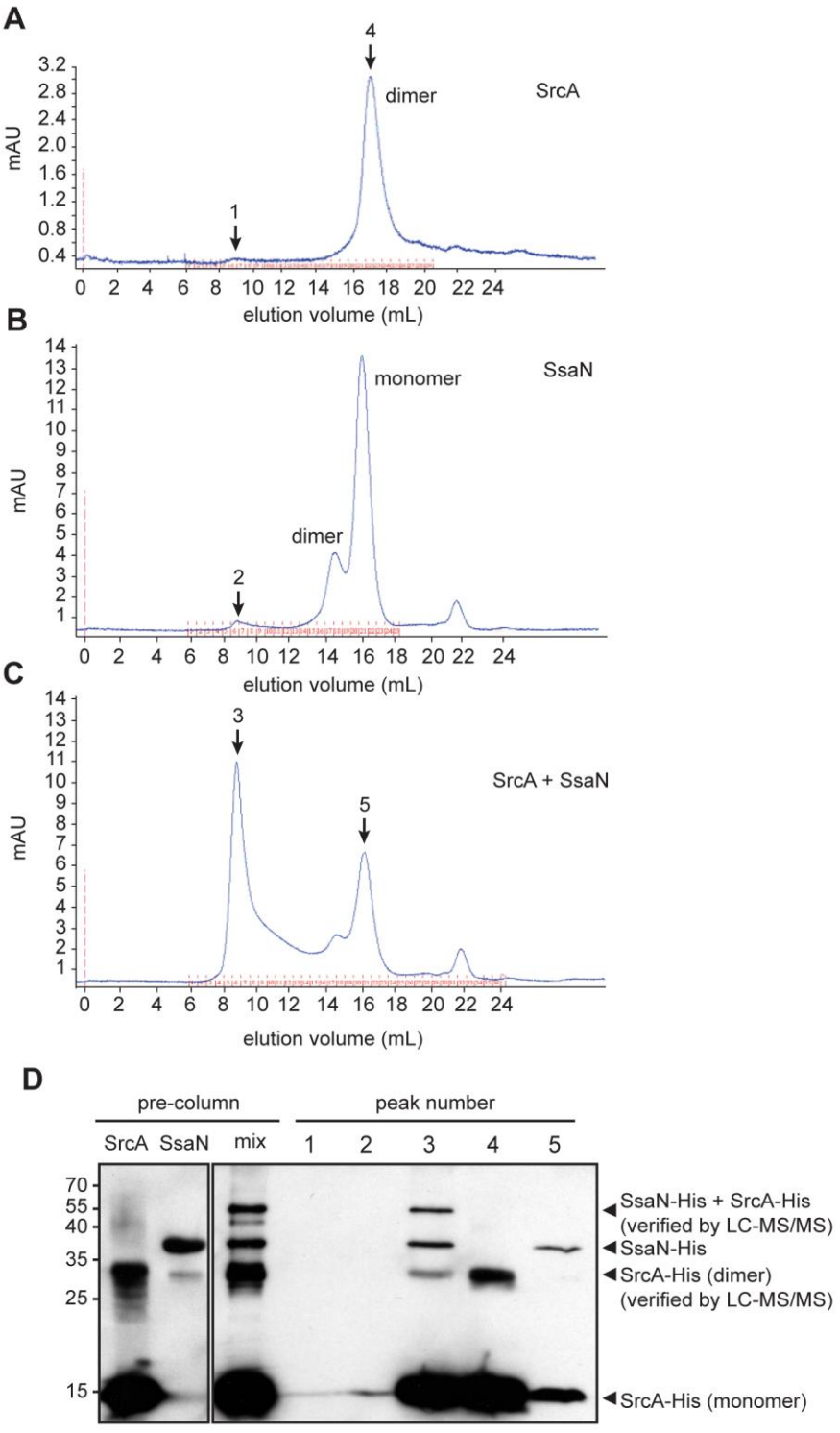

A
4
dimer
SrcA
mAU
1
elution volume (mL)
B
monomer
SsaN
dimer
2
C
3
SrcA + SsaN
5
D
pre-column
peak number
SrcA
SsaN
mix
1
2
3
4
5
70
55
40
35
25
15
SsaN-His + SrcA-His
(verified by LC-MS/MS)
SsaN-His
SrcA-His (dimer)
(verified by LC-MS/MS)
SrcA-His (monomer)

Figure 2.5. SrcA is a multi-cargo secretion chaperone for SseL and PipB2

(a) SILAC was used in conjunction with quantitative mass spectrometry-based proteomics for systematic identification of effector cargos bound by SrcA *in vivo*. **(b)** Mass:charge spectra for representative peptides (sequences shown) identified from SseL and OmpF, where normal isotope abundance-labeled peptides are indicated with open triangles and $^{2}H_{4}$-Lys or $^{13}C_{6}$-Arg labeled peptides are indicated by filled triangles. Protein abundance of SseL and PipB2 was examined by western blot in wild type and Δ*srcA* mutant cells from secreted **(c)** and cytosolic protein fractions **(d)**. Data is representative of 4 to 6 experiments.

Figure 2.5

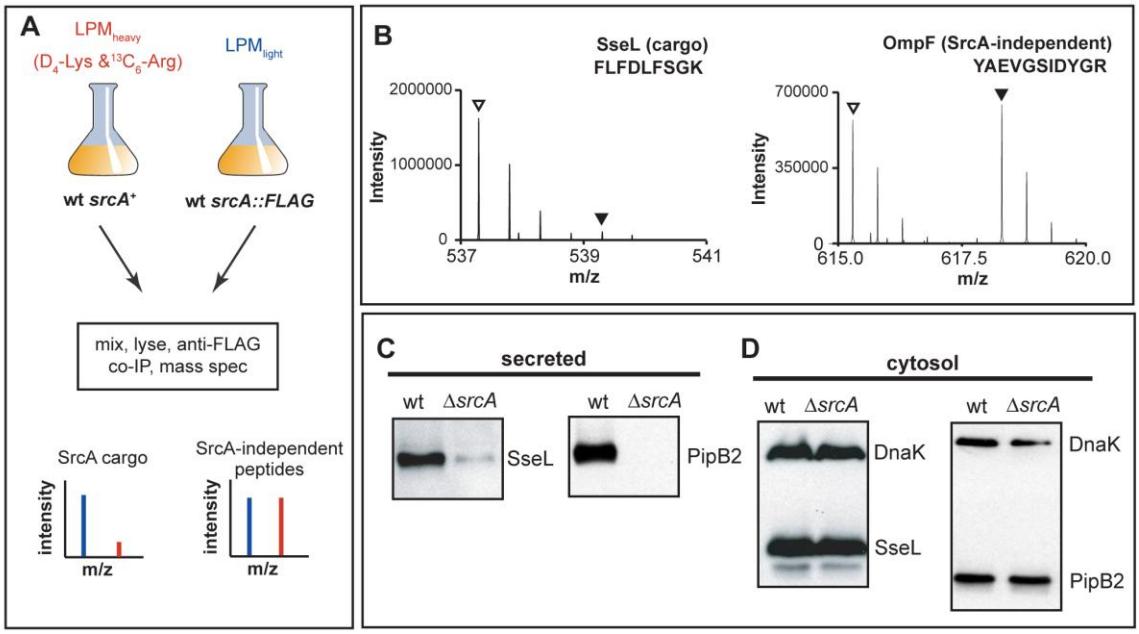
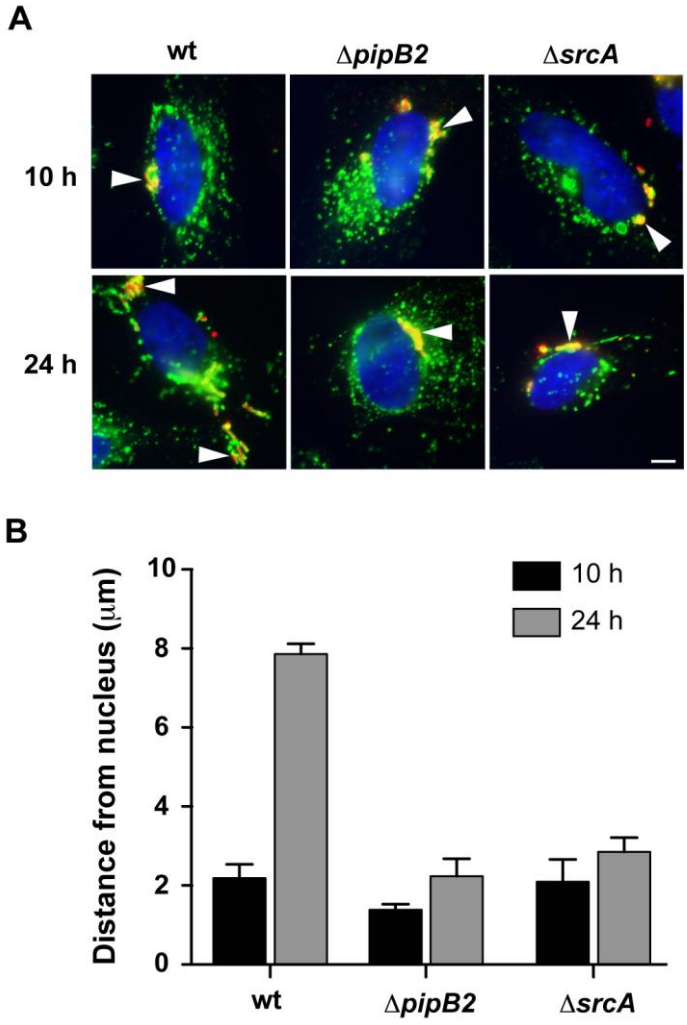

A
LPM heavy
(D4-Lys &13C6-Arg)
LPM light
wt srcA+
wt srcA::FLAG
mix, lyse, anti-FLAG
co-IP, mass spec
SrcA cargo
SrcA-independent
peptides
intensity
m/z
B
SseL (cargo)
FLFDLFSGK
OmpF (SrcA-independent)
YAEVGSIDYGR
Intensity
2000000
1000000
0
537
539
541
m/z
700000
350000
615.0
617.5
620.0
C
secreted
wt
ΔsrcA
SseL
PipB2
D
cytosol
DnaK
SseL
PipB2

Figure 2.6. SrcA is required for centrifugal movement of the SCV. (a) Intracellular positioning of SCVs containing wild type *Salmonella* or Δ*pipB2* and Δ*srcA* mutants after 10 and 24 h post-infection. HeLa cells were immunostained for bacteria (red) and LAMP1 (green), and the nucleus stained using DAPI (blue). Arrowheads indicate SCVs. Bar represents 10 µm. **(b)** HeLa cells were infected with wild type or Δ*pipB2* and Δ*srcA* mutants of *S.* Typhimurium. Cells were fixed at the indicated times, immunostained and analyzed as described in Methods. Means with average deviation for two separate experiments are shown.

Figure 2.6


A
wt
ΔpipB2
ΔsrcA
10 h
24 h
B
Distance from nucleus (μm)
10
8
6
4
2
0
10 h
24 h
wt
ΔpipB2
ΔsrcA

Supplementary Table 2.S1: Distribution of genetic loci in the Genus *Salmonellae*

Species (strain)	***srcA*[a]**	**SPI-1**	**SPI-2**	***pipB2***	***sseL***
S. enterica					
Typhimurium (LT2)	+ (100)	+	+	+ (100)	+ (100)
Typhimurium (SL1344)	+ (100)	+	+	+ (100)	+ (100)
Typhi (Ty2)	+ (97)	+	+	+ (95)	+ (93)
Typhi (CT18)	+ (97)	+	+	+ (95)	+ (93)
Paratyphi A	+ (97)	+	+	+ (98)	+ (93)
Choleraesuis	+ (99)	+	+	+ (98)	+ (93)
Newport (SL254)	+ (100)	+	+	+ (98)	+ (96)
Dublin (CT02021853)	+ (99)	+	+	+ (99)	+ (93)
Heidelberg (SL476)	+ (99)	+	+	+ (99)	+ (93)
Heidelberg (SL486)	+ (99)	+	+	+ (99)	+ (93)
Agona (SL483)	+ (98)	+	+	+ (94)	+ (94)
Schwarzengrund (SL480)	+ (98)	+	+	+ (94)	+ (91)
Schwarzengrund (CVM19633)	+ (98)	+	+	+ (94)	+ (91)
Enteriditis	+ (98)	+	+	+ (99)	+ (94)
Gallinarum	+ (98)	+	+	+ (99)	+ (94)
S. bongori	-	+	-	-	-

[a] +, present; -, absent (% identity to SL1344 orthologue)

Figure legend for Supplementary Figure 2.S1

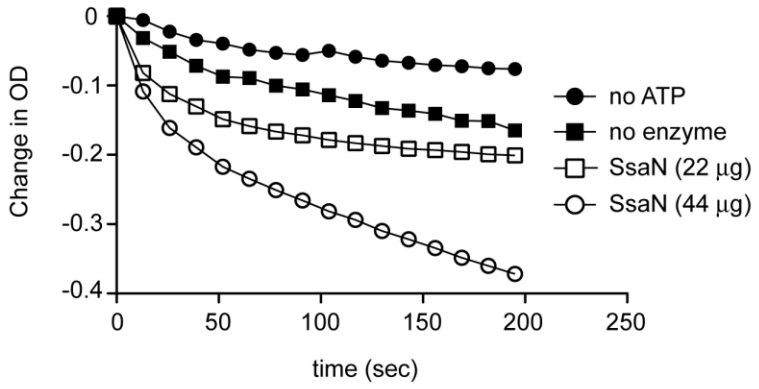
(a) Amino acid sequence alignment of SsaN and EscN from *E. coli*. The conserved Walker A and Walker B boxes from P-loop NTPases are shown in red and blue, respectively. Additional conserved catalytic residues are indicated in green including: Glu191 (conserved in position and orientation in EscN with F1 ATPase β-subunit); Arg352 (protrudes from adjacent monomer to bind ATP γ-phosphate in the ATP binding pocket of adjacent monomer); Gln412 (stabilizes ATP binding pocket in EscN). Residues correspond to SsaN numbering. **(b)** Purified SsaN has ATPase activity. SsaN activity was tested in a pyruvate kinase-lactate dehydrogenase coupled assay that monitors NADH oxidation coupled with ATP hydrolysis. Shown are representative data from three experiments.

Figure 2.S1

A

```
SsaN  MKNE---LMQRLRLKYP-------------PPDGYCRW-GRIQDVSATLLNAWLPGVFMG
EscN  MISEHDFVLER----YPRIQRVLNSTVPTLSLNSSTRYEGKITNIGGTIIKARLPKARIG
      * .*   :::*    **             . :.  *: *:* ::..*:::* ** . :*

SsaN  ELCCIKPGEELAEVVGINGSKALLSPFTSTIGLHCGQQVMALRRRHQVPVGEALLGRVID
EscN  AFYRIEPSQRLAEVIAIDEDEVFLLPFEHISGMYCGQWLSYQGDEFKIRVGDELLGRLVD
       :  *:*.:.****:.*: .:.:* **    *::*** :     ..:: **: ****::*

SsaN  GFGRPLDGR----ELPDVCWKDYDAMPPPAMVRQPITQPLMTGIRAIDSVATCGEGQRVG
EscN  GIGRPMGSNITAPYLP--FERSLYAEPPDPLLRQVIDQPFTLGVRAIDGLLTCGIGQRIG
      *:***:...     **    :.  * ** .::** * **:  *:****.: *** ***:*
        Walker-A
SsaN  IFSAPGVGKSTLLAMLCNAPDADSNVLVLIGERGREVREFIDFTLSEETRKRCVIVVATS
EscN  IFAGSGVGKSTLLGMICNGASADIIVLALIGERGREVNEFLAL-LPQSTLSKCVLVVTTS
      **:..********.*:**...**  **.*********.**: : *.:.* .:**:**:**
                                Walker-B
SsaN  DRPALERVRALFVATTIAEFFRDNGKRVVLLADSLTRYARAAREIALAAGETAVSGEYPP
EscN  DRPALERMKAAFTATTIAEFFRDQGKNVLLMMDSVTRYARAARDVGLASGEPDVRGGFPP
      *******::* *.**********:**.*:*: **:********::.**:**. * * :**

SsaN  GVFSALPRLLERTGMGEKGSITAFYTVLVEGDDMNEPLADEVRSLLDGHIVLSRRLAERG
EscN  SVFSSLPKLLERAGPAPKGSITAIYTVLLESDNVNDPIGDEVRSILDGHIVLTRELAEEN
      .***:**:****:* . ******:****:*.*::*:*:.*****:*******:*.***..

SsaN  HYPAIDVLATLSRVFPVVTSHEHRQLAAILRRCLALYQEVELLIRIGEYQRGVDTDTDKA
EscN  HFPAIDIGLSASRVMHNVVTSEHLRAAAECKKLIATYKNIELLIRIGEYTMGQDPEADKA
      *:****:  : ***:  *.: ** : **  :: :* *:::**********  * *.::***

SsaN  IDTYPDICTFLRQSKDEVCGPELLIEKLHQILTE
EscN  IKNRKAIQSFIQQSTKDISSYEKTIESLFKVVA-
      *..   * :*::**..::.. *  **.*.::::
```

B



Cooper et al. Supplementary Figure 1

Supplementary Methods (Cooper et al.)

For standard LPM minimal medium, the final concentration of the various components is:
5 mM KCl
7.5 mM (NH4)2SO4
0.5 mM K2SO4
80 mM MES
38 mM glycerol (0.3% v/v)
Amino acids (1/100 dilution of a mixture containing the [amino acids] in 10% casaminoacids (see below)
24 mM MgCl2
500 mM PO43- (added as mixture of mono- and dibasic phosphate)

The first 4 ingredients are prepared as a 5 X salts solution and autoclaved to sterilize. To prepare the working medium, the salts solution, MgCl2, phosphate ions, glycerol and casamino acids are combined. The pH is adjusted to 5.8 and the medium is filter sterilized prior to use.

For SILAC minimal medium, the casamino acid component of LPM is replaced with individual amino acids as follows:

	% of dry cas	Weight (g) for 100 ml stock	Molarity to mock aa in 10% [0.1%] cas (mM)
Alanine	3.0	0.30	34 [0.34]
Asparagine	0.0	0.0	0.0 [-]
Aspartic acid	2.4	0.24	18 [0.18]
Cysteine	0.1	0.01	0.83 [0.0083]
Glutamic acid	15.9	1.59	108 [1.08]
Glutamine	0.0	0.0	0.0 [-]
Glycine	1.4	0.14	19 [0.19]
Histidine	0.8	0.08	5.2 [0.052]
Isoleucine	4.0	0.40	30 [0.30]
Leucine	5.0	0.50	38 [0.38]
Methionine	1.4	0.14	9.4 [0.094]
Phenylalanine	3.6	0.36	22 [0.22]
Proline	8.0	0.80	69 [0.69]
Serine	2.1	0.21	20 [0.20]
Threonine	1.5	0.15	13 [0.13]
Tryptophan	0.0	0.0	0.0 [-]
Tyrosine	0.4	0.04	2.2 [0.022]
Valine	5.6	0.56	48 [0.48]
Arginine*	2.5	0.25	14 [0.14]
Lysine*	5.2	0.52	36 [0.36]

*omit arginine and lysine from the stock when labeling for SILAC

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Chapter III - The *Salmonella* pathogenicity island-two encoded virulence chaperone SscA and the translocon component SseC are required for virulence and regulation of SPI-2 encoded genes in *Salmonella enterica*

Chapter III – Co-authorship statements

The following experiments were performed by collaborators other than myself:

1) Cell culture infections were performed and analyzed by David T Mulder.

Abstract

Salmonella enterica is a causative agent of food borne gastroenteritis and the systemic disease known as typhoid fever. This bacterium employs two type three secretion systems (T3SS) to secrete proteins termed effectors into host cells to manipulate cellular function. The *Salmonella* pathogenicity island two (SPI-2) is composed of 32 genes and encodes a T3SS, virulence chaperones and effectors. Correct assembly and function of the T3SS requires cytosolic virulence chaperones that direct apparatus, translocon, and effector proteins to the cytosolic face of the T3SS. Despite the extensive identification of effectors in *S. enterica* , including over twenty effectors encoded outside of SPI-2, only three virulence chaperones have been identified to date. Here we report that the SPI-2 encoded protein, SscA, is a class II virulence chaperone for the translocon component SseC. Furthermore, we report a role for both these proteins in the transcriptional regulation of SPI-2. These data expand our knowledge of chaperone-cargo pairs for the SPI-2 T3SS and identify a novel regulatory input.

Introduction

Exploitation of new host niches requires bacterial pathogens to evolve intricate virulence strategies to promote their survival. One such strategy, employed by the Gram-negative bacterium *Salmonella enterica* is the translocation of effector proteins from the bacterial to host cytosol through the activity of type three secretion systems (T3SSs). *S. enterica* has two T3SSs, encoded within *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2, which facilitate invasion of and intracellular survival within host cells respectively (Groisman & Ochman, 1993, Shea et al., 1996). The formation of T3SSs is complex, as these molecular machines must form a channel in the bacterial inner and outer membrane, a needle like filament which extends toward the host cell, and a translocon pore that imbeds within the host cell membrane. Once T3SSs are formed, virulence chaperones direct effectors to the cytoplasmic face of the T3SS where they are unfolded and translocated into the host cell cytoplasm to modulate cellular function.

Virulence chaperones involved in T3SS assembly and function fall under three classes: class I virulence chaperones deliver effectors (IA for a single effector, or IB for multiple), class II chaperones deliver translocon components (both the translocon platform and membrane embedded ring components), and class III chaperones deliver apparatus components (Cornelis, 2006). Virulence chaperones are essential proteins present in all Gram-negative bacteria harbouring a T3SS. They often possess high structural similarity, they are generally low in amino acid sequence conservation. Virulence chaperones are therefore often identified based on their physical characteristics: Class I possess an acidic pI, have low (~9-15 kDa) molecular weight and function as dimers adopting a horseshoe-like shape (Cooper et al., 2010, Luo et al., 2001, Stebbins & Galan, 2001). Class II virulence chaperones also form dimers, yet do not have

an acidic pI, possibly due to the differences in substrate binding (Buttner et al., 2008, Yip et al., 2005).

Virulence chaperones are not limited to substrate binding and targeting, but may also play a role in gene regulation, further underlining the importance of their identification and characterization. In the Gram-negative flagellar system and the *Yersinia* virulence plasmid encoded T3SS, loss of virulence chaperones result in decreased expression of apparatus components from their parent virulence clusters (Parsot et al., 2003). In the structurally analogous flagellar system, the FlgN chaperone interacts with FlgKL to form a repressive complex which inhibits expression of late flagellar genes (Bennett et al., 2001). The *Yersinia* virulence plasmid encodes the virulence chaperone SycD (also known as LcrH) which is responsible for aiding in the secretion of the translocon components YopB and YopD, the latter responsible for establishing a negative feedback loop, preventing effector gene expression at early stages of infection (Francis et al., 2001). As SycD is required for YopD stability in the cytosol, both chaperone and cargo are necessary for proper coordination of Yop expression.

In *S. enterica*, the identification and classification of virulence chaperones involved in SPI-2 T3SS function is incomplete. Although over twenty SPI-2 effectors have been characterized, only two class I virulence chaperones have been identified, SrcA and SscB (Cooper et al., 2010, Dai & Zhou, 2004). The SPI-2 encoded class II virulence chaperone, SseA, is responsible for the secretion of the putative translocon platform component SseB and part of the translocon SseD, but not the second translocon component SseC (Coombes et al., 2003, Ruiz-Albert et al., 2003, Zurawski & Stein, 2003). In light of these findings, the identification and characterization of a virulence chaperone necessary for secretion of the translocon component SseC was examined. In this study we demonstrate that the SPI-2 encoded virulence chaperone

SscA directs secretion of SseC, and not SseD or SseB. Additionally, SscA was shown to interact with SseC under SPI-2 inducing conditions. In cell culture and mouse infections we show that both SscA and SseC are required for virulence. Furthermore, we identify a regulatory role for SseC and SscA in transcriptional regulation at SPI-2 promoters.

Materials and Methods

Bacterial strains, DNA manipulations, and growth conditions

Salmonella enterica serovar Typhimurium strain SL1344 (*S.* Typhimurium) was used as a wild type stain and for development of all subsequent strains. Genomic mutations were performed as described previously (Datsenko & Wanner, 2000). LPM media was used to induce the expression of SsrB regulated genes (Coombes et al., 2005).

Co-immunoprecipitation of SscA and SseC

Co-immunoprecipitations were performed with *S.* Typhimurium harbouring the inducible pCTC-FLAG plasmid (Sigma) encoding *sscA::FLAG* using M2-agarose beads conjugated with anti-FLAG antibodies (F-gel) (Sigma). Strains were grown overnight in LB and sub-cultured 1:50 into LPM for growth to an optical density of 0.6 at 600nm. Cultures were then centrifuged at 3000g for 10 minutes, and re-suspended in PBS containing mini-EDTA protease inhibitor tablets (1 tablet / 50ml) (PBS-PI) (Roche). Cells were then lysed by sonication for 30 seconds, six times, with 60 second intervals between sonications (Misonix Sonicator 3000, Misonix). To obtain cytosolic protein, lysates were pelleted at 3000g for 15 minutes at 4 degrees Celsius with supernatant removed as cytosolic protein fraction. F-gel was equilibrated with PBS-PI containing 10 µg/ml BSA for 60 minutes at 4 degrees Celsius rocking, and washed with PBS-PI

three times before adding cytosolic protein and incubating for 16 hours at 4 degrees Celsius rocking. The unbound protein fraction was removed by pelleting the F-gel at 1000g for 5 minutes and removing the supernatant. The F-gel was then washed ten times with PBS-PI containing 0.1% Triton-X 100 before eluting the proteins with SDS-sample buffer (1 M Tris pH 8.0, 20% SDS, 0.5 M EDTA pH 8, 10% glycerol, 200 mM dithiothreitol). Standard Western blot techniques were used to visualize protein fractions with antibodies directed towards SseC (gift from Michael Hensel) or the FLAG epitope (Sigma).

Analysis of secreted proteins from bacterial cultures

Strains were grown overnight in LB and sub-cultured 1:50 into LPM for growth to an optical density of 0.6 at 600nm. Cultures were then centrifuged for 2 min at 10 000g, to obtain secreted protein from the supernatants which were filtered through a 0.2µm pore filter (Pall Scientific) and concentrated with trichloro-acetic acid at a final concentration of 10%. Protein suspensions were then centrifuged at 16000g at 4 degrees Celsius for 30 minutes before washing with acetone and re-suspending in SDS-sample buffer to represent the secreted protein fraction. To ensure equal protein concentrations, both cytosolic and secreted protein samples were re-suspended according to growth OD. Standard Western blotting techniques were used with antibodies directed at DnaK (Convance), SseC (Gift from Michael Hensel), SseB, and SseD (Gift from John Brumell).

Cell culture methods

Experiments were performed independently in triplicate, twice in 24 well plates seeded with $2x10^5$ RAW 264.7 cells. Bacterial overnight cultures were washed and diluted in DMEM/10%

FBS and macrophages were infected at a MOI of 50 for 30 minutes then washed 3 times in PBS. Cells were incubated at 37 degrees Celsius with 5% CO_2. Media was replaced with DMEM/10% FBS/100 ug/mL gentamicin, followed by a 75 minute incubation to kill extracellular bacteria. Wells were washed twice with PBS, at 2 hour and 20 hour time point RAW cells were lysed with lysis buffer (1% Triton X-100, 0.1% SDS in PBS) to release intracellular bacteria. CFU per ml was determined by serial dilution and spotting on to agar plates.

Mouse infections

Animal protocols were approved by the Animal Research Ethics Board at McMaster University and in accordance to guidelines from the Canadian Council on the Use of Laboratory Animal. Competitive infections (CI) were performed in female C57BL/6 mice (Charles River) by oral inoculation of equal amounts of marked wild type and mutant strains as described previously (Cooper et al., 2010). CI was calculated as CFU $(\text{mutant/wild type})_{output}/(\text{mutant/wild type})_{input}$ in the spleen, liver, and cecum of each mouse.

Transcriptional reporter assays

Indicated SPI-2 promoter regions were cloned into the pGEN plasmid as described previously (Osborne & Coombes, 2011). Strains were grown overnight in LB and sub-cultured 1:50 into LPM in black clear bottom 96 well plates. At given time points, luminescence values were read using a luminometer (Envision, Perkin Elmer), with values normalized to OD_{600} and expressed as a ratio of promoter expression in mutant background over wild type.

Results

Identification of SscA as a virulence chaperone

SscA was identified as a SPI-2 encoded candidate virulence chaperone. At 18kDa, the small size of SscA is consistent with other chaperones, as well as its sequence similarity (46% conserved residues) to SycD, a translocon chaperone in *Yersinia*. Using the structure of SycD (PDB 2VGY), as a model, putative domains within SscA were mapped (Altschul et al., 1997). SscA was predicted to be a solely α-helical protein, possessing eight α-helices and a large tetratricopeptide repeat (TPR) domain from amino acids 36 to 137 (Buttner et al., 2008) similar to that observed in SycD (Figure 3.1).

The putative virulence chaperone SscA interacts with the translocon protein SseC

Chaperones exert their biological function through a physical interaction with their substrate cargo. To examine whether SscA is a chaperone for SseC, we tested the ability for SscA to interact with SseC during growth in SPI-2 inducing conditions. An SscA::FLAG construct was used for co-immunoprecipitation, and SseC was found in eluted samples visualized with western blotting (Figure 3.2). This interaction was not observed in the wild type strain suggesting a specific protein-protein interaction.

SscA is required for secretion of SseC

To determine whether the physical interaction between SscA and SseC was the result of a functional virulence chaperone interaction, we examined the secretion of SseC in an *sscA* mutant background under SPI-2 inducing conditions. The secreted protein fraction of both wild type *S.* Typhimurium and a strain harbouring a mutation for *sscA*, were probed for the presence of

translocon proteins SseB, SseC, and SseD. The *sscA* mutant was unable to secrete SseC into the culture supernatant whereas SseC was secreted by wild type cells. Although the *sscA* mutant failed to secrete SseC, there was abundant SseC protein in the bacterial cytosol, indicating that while equal amounts of the protein are present in the cytosol, a secretion defect exists rather than expression defect. Equal amounts of SseB and SseD were found in both secreted and cytosolic fractions, indicating that SscA has no effect on the regulation or secretion of these proteins (Figure 3.3).

SseC and SscA are required for fitness in vivo

Given our finding that SscA is required for secretion of the translocon component SseC, we examined the importance of these proteins during infection. Loss of either *sscA* or *sseC* significantly reduced intracellular replication in RAW264.7 macrophages by twenty hours post infection compared to the wild type strain (Figure 3.4A). Interestingly, replication was decreased to 10% in the *sseC* mutant, but only 50% with the *sscA* mutant suggesting additional functions for *sseC*. To determine whether similar phenotypes were seen in a mammalian infection model, mice were orally infected with a mixed inoculum containing equal proportions of wild type and mutant bacterial cells and competitive fitness was determined. After 72 hours of infection, the spleen, liver, and cecum were homogenized and plated to determine wild type and mutant strain CFU. The competitive indices for both *sseC* and *sscA* mutant strains yielded values below 0.2 indicating strong virulence attenuation (Figure 3.4B-C). These results indicate that both SseC and SscA are critical to infection both in cell culture and animal models.

Loss of either sscA or sseC results in altered expression from SPI-2 encoded promoters

To determine whether SscA and SseC have an effect on SPI-2 regulation, SPI-2 promoters were cloned into a transcriptional luminescence reporter plasmid and were examined for expression under SPI-2 inducing conditions in either an *sscA* or *sseC* mutant background. Expression of the apparatus promoter upstream of *ssaG* decreased in the *sseC* mutant background to less than 10% of wild type expression prior to the 2 hour time point, where expression then remained constant. A similar result was seen for expression at the apparatus promoter upstream of *ssaR* in the *sseC* mutant background, where expression was 40-80% lower than wildtype (Figure 3.5). Expression from each apparatus promoter was higher in the *sscA* mutant relative to wild type. The effector promoter(*sseE)* was unaffected by the *sscA* mutant background but increased from 20 to 40 fold at later time points in the *sseC* mutant background.

Discussion

Protein-chaperone interactions are essential for the function of T3SSs due to their ability to target apparatus and effector proteins to the T3SS. Class II virulence chaperones are particularly important as they are responsible for the secretion of the translocon, necessary for T3SS to imbed within the host cell membrane and for the subsequent translocation of effectors. Given the knowledge of the class II chaperone SycD of *Yersinia*, we hypothesized that SscA and SycD would be functionally analogous based on their high sequence conservation. Residues predicted to be involved in protein cargo interaction in SycD are conserved in SscA. The structure of SycD forms a crescent-like shape, with the concave area possessing protein interaction sites which are shared between SycD and SscA (Y40, Y52, Y93) (Buttner et al., 2008). Additionally, the *Shigella* class II virulence chaperone IpgC possesses a similar structure, with the concave

face shown to bind a peptide of its substrate IpaD in a co-crystal representation (Lunelli et al., 2009). This suggests that a common cargo binding region may exist between these class II virulence chaperones. The ten amino acids at the C-terminus in SycD are not found in SscA, leaving this area as the most unconserved region, however no function has been predicted for the region, either in dimerization or protein interaction.

We identified SscA as a class II virulence chaperone which interacts with the translocon SseC. We demonstrated through cell culture and mouse model infections that deletion of *sseC* or *sscA* strongly decreased the fitness of *S. enterica*. As documented previously, effectors may be secreted from the cell in the absence of the translocon, however translocation of effector proteins such as SspH2 into the host cell require a translocon, and absence of the translocon results in a virulence defect (Nikolaus et al., 2001, Sory & Cornelis, 1994). Interestingly, the *sseC* mutant has a much more pronounced negative effect on replication in RAW264.7 cells. This suggested an additional virulence role for SseC in cell culture infections. However, when these mutants were compared for their abilities to disseminate to systemic sites *in vivo* both mutants were comparably deficient.

In *Yersinia*, YopB and YopD form a heterologous translocon complex, with additional regulatory properties belonging to YopD. *S. enterica* lacks a YopD orthologue and these regulatory properties apparently belong to SseC, a YopB orthologue (53% conserved amino acid sequence). Nevertheless, the negative feedback regulation on *Yersinia* effectors is similar to the regulatory effect SseC has on the *sseE* promoter (Francis et al., 2001, Chen & Anderson, 2011, Olsson et al., 2004). The assembly of the T3SS must occur before effectors can be secreted, therefore the last piece of the T3SS to be assembled, the translocon, would be the most logical candidate for a repressor for effector secretion. Once the translocon is secreted, its absence in

the bacterial cytosol would relieve repression on effector gene expression. What differs between the regulation profiles of YopB and SseC is that SseC also appears to be responsible for upregulating structural genes of the T3SS at the *ssaG* and *ssaR* promoters. This fits the above proposed model in that accumulation of cytosolic SseC may result from its inability to be secreted, possibly due to an incomplete apparatus, and thus a need for more apparatus gene expression would exist. Another striking difference between the *Yersinia* and *S. enterica* translocon regulation networks is the necessity for their virulence chaperones for cytoplasmic stability. Both YopB and YopD require SycD for cytoplasmic stability, and in the case of YopD, the ability to regulate Yop genes. In contrast, preliminary studies suggest that SscA is dispensable for SseC stability and has less effect on the regulation of SPI-2 encoded promoters. This leads to the hypothesis that SseC binds additional proteins such as a DNA binding complex for stability. Additionally, other factors shown to bind the YopD-SycD complex include LcrV, YscM1 and YscM2, for which no orthologues are found in *Salmonella* (Cambronne & Schneewind, 2002). The lack of additional orthologues in *Salmonella* indicates a perhaps more complex picture of regulation at the SPI-2 T3SS. It is fascinating that in both systems a translocon component is needed for regulation of the T3SS encoding genes, however which member of the translocon accomplishes this is not conserved.

In summary, we have identified a novel SPI-2 class II virulence chaperone and its translocon protein cargo. These findings demonstrate that establishment of a functional SPI-2 T3SS translocon is dependent on at least two class II chaperones, SscA and SseA. Additionally, we identify a novel regulatory feedback mechanism for the SPI-2 T3SS wherein translocon component levels control expression levels at apparatus and effector promoters. The importance of this protein pair at both the secretory and regulatory levels make it an interesting virulence

mechanism associated with *S. enterica* pathogenesis. Additional research should centre around the mechanisms used for translocon regulation at pathogenicity islands encoding T3SS, as key elements essential for T3SS function must be in play.

Figure 3.1 – Sequence analysis for virulence chaperone SycD and putative virulence chaperone SscA. Conserved alpha helical regions are overlaid with blue bars. Alignment was performed with Clustal W software (www.ebi.ac.uk), alpha helix content was obtained from the published SycD crystal structure (PDB 2VGY) and from SSpro8 software (www.ics.uci.edu).

Figure 3.1

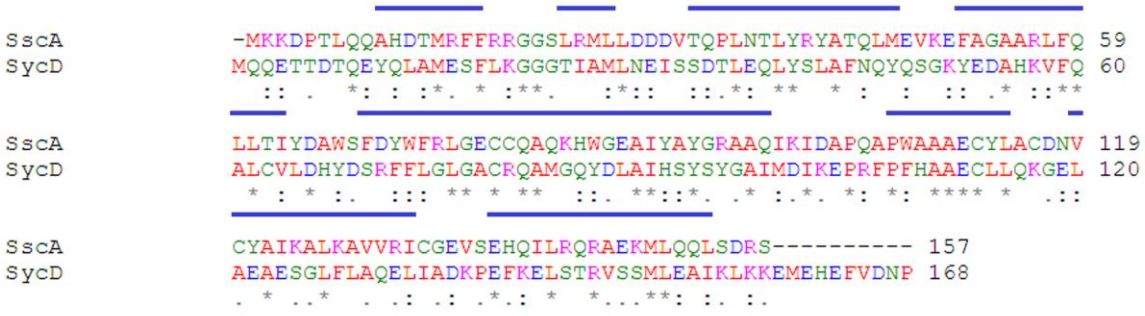

SscA -MKKDPTLQQAHDTMRFFRRGGSLRMLLDDDVTQPLNTLYRYATQLMEVKEFAGAARLFQ 59
SycD MQQETTDTQEYQLAMESFLKGGGTIAMLNEISSDTLEQLYSLAFNQYQSGKYEDAHKVFQ 60
SscA LLTIYDAWSFDYWFRLGECCQAQKHWGEAIYAYGRAAQIKIDAPQAPWAAAECYLACDNV 119
SycD ALCVLDHYDSRFFLGLGACRQAMGQYDLAIHSYSYGAIMDIKEPRFPFHAAECLLQKGEL 120
SscA CYAIKALKAVVRICGEVSEHQILRQRAEKMLQQLSDRS---------- 157
SycD AEAESGLFLAQELIADKPEFKELSTRVSSMLEAIKLKKEMEHEFVDNP 168

Figure 3.2 – The virulence chaperone SscA interacts with the translocon protein SseC. Wild type (Wt) and a strain harboring a plasmid expressing SscA::FLAG were grown in SPI-2 inducing conditions, lysed, and subjected to immunoprecipitation with anti-FLAG agarose beads and western blotting. (U – unbound protein fraction from initial incubation, FW – Final wash, E – elution).

Figure 3.2

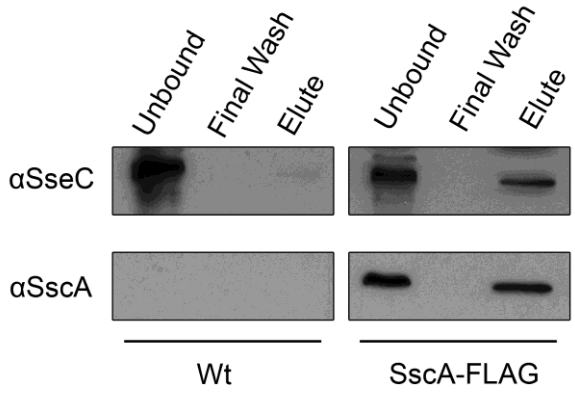

Unbound
Final Wash
Elute
Unbound
Final Wash
Elute
αSseC
αSscA
Wt
SscA-FLAG

Figure 3.3 – SscA is required for the secretion of SseC. Cytosolic and secreted fractions for wild type (Wt) and a *sscA* mutant were probed for SseB, SseC, and SseD. DnaK was used as a cytoplasmic loading control, and to ensure no cytoplasmic protein was present in secreted fractions (not shown).

Figure 3.3

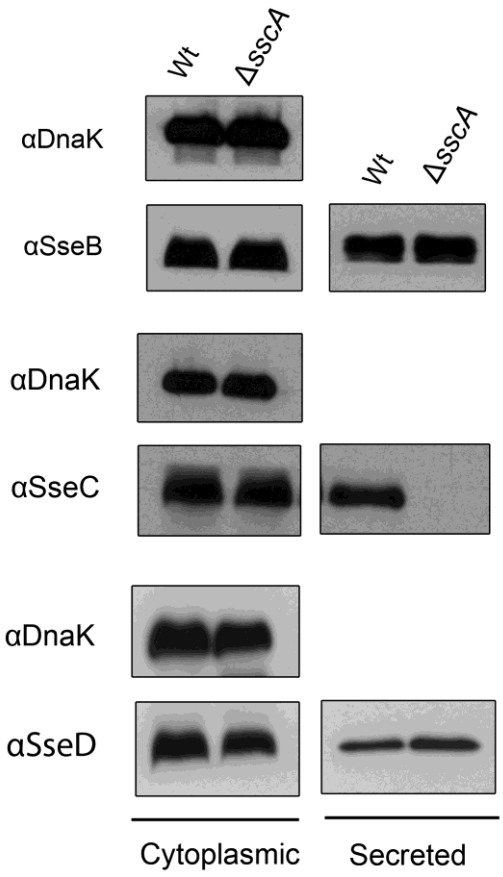

Wt
ΔsscA
Wt
ΔsscA
αDnaK
αSseB
αDnaK
αSseC
αDnaK
αSseD
Cytoplasmic
Secreted

Figure 3.4 –SscA and SseC are required for fitness *in vivo*. (A) RAW 264.7 cells were infected with indicated mutant strains and lysed at 2 and 20 hour time points to determine mean fold replication normalized to wild type (Wt) infections. Error bars represent standard error for three infections. (B-C) Mutant strains of *sscA* and *sseC* were generated and grown in LB before inoculating 5-6 week old C57BL6 mice with approximately $3x10^7$ CFU. Each data point represents an individual animal.

Figure 3.4

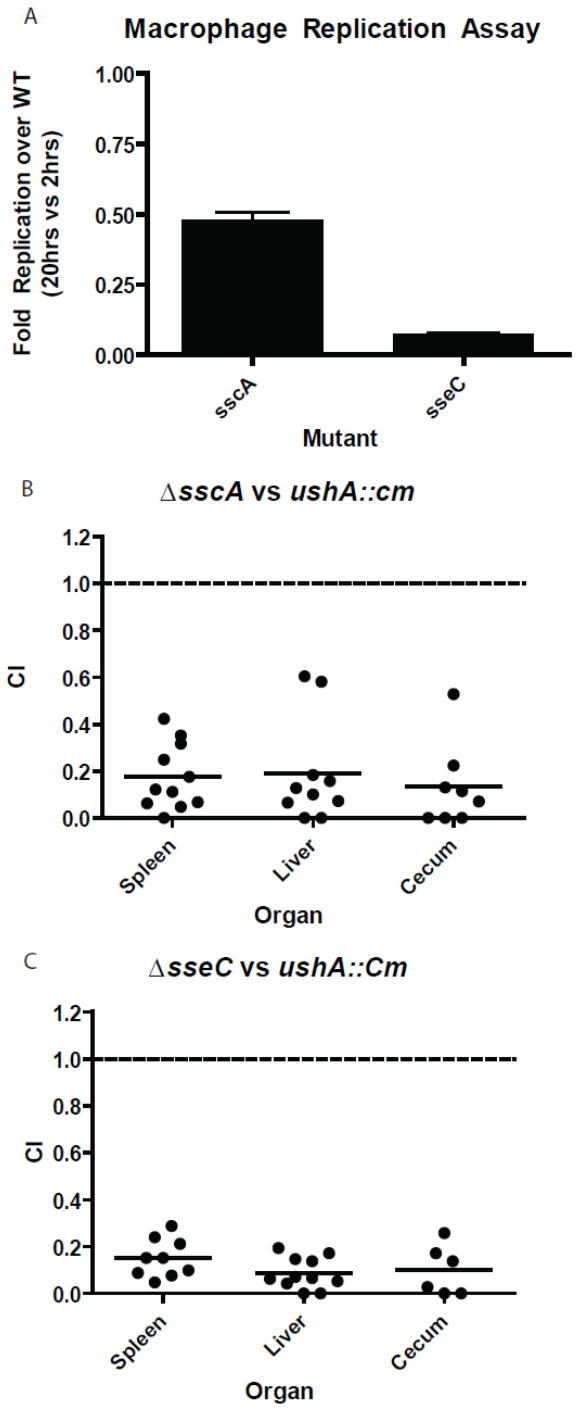
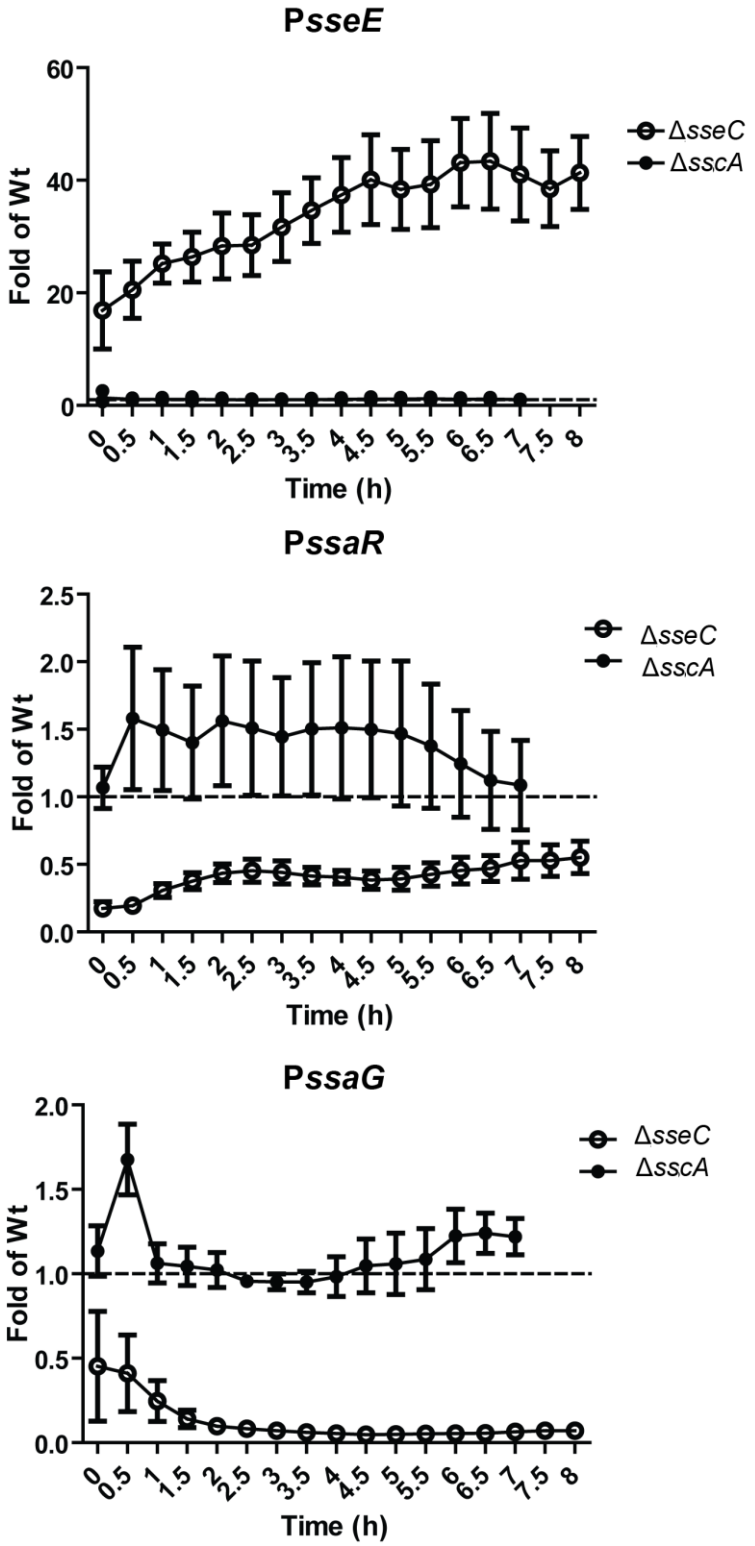

A
Macrophage Replication Assay
Fold Replication over WT (20hrs vs 2hrs)
1.00
0.75
0.50
0.25
0.00
sscA
sseC
Mutant
B
ΔsscA vs ushA::cm
CI
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Spleen
Liver
Cecum
Organ
C
ΔsseC vs ushA::Cm
CI
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Spleen
Liver
Cecum
Organ

Figure 3.5 – Effect of SseC and SscA on SPI-2 promoter expression. Indicated SPI-2 promoters were cloned into a lux reporter plasmid and grown in indicated mutant backgrounds and wild type (Wt). Luminescence was measured and normalized to OD_{600} at 30 min time points, and expressed as a ratio to wild type values.

Figure 3.5


PsseE
60
40
20
0
Fold of Wt
ΔsseC
ΔsscA
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
Time (h)
PssaR
2.5
2.0
1.5
1.0
0.5
0.0
Fold of Wt
ΔsseC
ΔsscA
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
Time (h)
PssaG
2.0
1.5
1.0
0.5
0.0
Fold of Wt
ΔsseC
ΔsscA
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
Time (h)

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Chapter IV - Functional and structural characterization of the *Salmonella* pathogenicity island-two encoded type three secretion system ATPase SsaN

Chapter IV – Co-authorship statements

The following experiments were performed by collaborators other than myself:

1) Protein purification, and crystal generation was performed by Kun Zhang.

2) Diffraction, data collection, and refinement of the SsaN crystal structure was performed by Seiji Sugiman-Marangos and Murray S Junop.

Abstract

Bacterial pathogens often employ secretion systems to modify host cell function. The type 3 secretion system (T3SS) is composed of apparatus proteins that form a bridge between bacterial and host cell cytoplasm. At the base of the T3SS, virulence chaperones bound to effector proteins dock at an ATPase protein, where the effector-chaperone complex is uncoupled and the secretion of the effector begins. Here we report the crystal structure of the *Salmonella enterica* T3SS ATPase SsaN. SsaN resembles other T3SS ATPases, however different conformations were observed suggesting that this family of proteins adopt altered structural states. Additionally, mutation of charged residues within the central channel of the proposed hexamer model decreases secretion. In an effort to dissect the function of the N-terminal domain of SsaN, a truncation analysis coupled to membrane localization and effector secretion experiments was conducted, concluding that the N-terminus is not required for membrane localization, or effector secretion. These findings contribute to the understanding of T3SS ATPase function, and apparatus formation at the proximal end of the SPI-2 encoded T3SS.

Introduction

The T3SS is a common system utilized by Gram-negative bacterial pathogens. It is composed of oligomeric protein rings that interact to create a channel spanning the inner, outer, and host cell membrane (Worrall et al., 2011). Once assembled, the T3SS acts as a bridge for secreted proteins termed effectors, which are injected into the host cell to modify function and enhance bacterial survival. The process of effector secretion relies on the binding of virulence chaperones to generate an effector-chaperone complex, which shuttle effectors to the base of the T3SS. Once docked with the T3SS, an ATPase protein dissociates the effector from the virulence chaperone in an ATP dependent manner, as well as unfolding the effector to compact the protein within the T3SS channel for secretion (Akeda & Galan, 2005). Based on structural similarities, the F_1F_0 α and β subunits of the ATP synthase complex may be an ancestral protein complex of the T3SS ATPase, however how they have evolved into a virulence mechanism has yet to be assessed.

Of the many conserved T3SS ATPases, structural data is available for only two proteins, the LEE encoded EscN of enteropathogenic *E. coli* (EPEC), and the flagellar component FliI (Imada et al., 2007, Zarivach et al., 2007). The structure obtained for EscN contained a 102 amino acid N-terminal truncation lacking a predicted β-barrel domain thought to mediate protein-protein interaction, oligomer stabilization, and membrane localization. The structural resemblance to the β-subunit of the F_1F_0 ATP synthase was confirmed when EscN was modelled as a hexamer. Oligomerization studies with EscN previously showed that a hexamer is formed *in vitro* (Andrade et al., 2007). The inner channel of the EscN hexamer model ranges from 18 to 50 Å, indicating that should effectors pass through the hexamer and subsequent T3SS rings, the effector would require unfolding. The second T3SS ATPase to be crystallized was FliI of the

highly conserved flagellar system (Imada et al., 2007). FliI shares both sequence and structural similarities to EscN, however full-length FliI was crystallized, displaying an N-terminal six stranded β-barrel domain not seen in the truncated EscN structure. The first 100 amino acids are not as well conserved compared with the full length protein comparison of FliI and EscN (48% conserved residues versus 60%), suggesting this domain may not have a conserved function in the different systems. Consistent with this idea, the accessory protein FliH of the flagellar system has been shown to interact with this N-terminal domain, while no such orthologue has been identified in EPEC (Minamino & MacNab, 2000).

Perhaps one of the most functionally characterized T3SS ATPases is the *Salmonella* pathogenicity island-1 (SPI-1) encoded InvC. Using biochemical studies, InvC has been shown to localize with the membrane fraction and other T3SS proteins, interact with effector chaperone complexes, and dissociate these complexes while unfolding effectors prior to secretion (Akeda & Galan, 2004, Akeda & Galan, 2005). Additionally, the *Pseudomonas* T3SS ATPase HrcN was shown to oligomerize into a hexamer when associated with the membrane (Pozidis et al., 2003), and may actually form a double hexamer structure as seen with cryo-electron microscopy (Muller et al., 2006). To date no information exists for the function of residues lining the modelled hexameric channel of T3SS ATPases, the precise function of the N-terminal domain, or T3SS ATPase orientation with respect to the T3SS.

Here we report the crystal structure of the *Salmonella* pathogenicity island-2 (SPI-2) T3SS ATPase SsaN in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) at a resolution of 2.1Å. Three regions within the proposed hexameric channel were analyzed. One region in particular demonstrated decreased effector secretion following a R334W amino acid substitution. Additionally, we show that *ssaN* is required for fitness in a mouse infection model.

Furthermore, the N-terminal domain was found to be dispensable for effector secretion and membrane localization with the T3SS.

Materials and Methods

Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium SL1344 was used as a parent strain for any genetic manipulations. Mutant generation was performed as described previously (Datsenko & Wanner, 2000), and standard cloning techniques were used for plasmid construction, including overlap extension PCR for *ssaN* point mutant generation. To induce SPI-2 and related gene expression, LPM media was used, sub-culturing strains 1:50 from overnight LB broth cultures (Coombes et al., 2004).

Competitive infections

All animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care. The Animal Research Ethics Board at McMaster University approved all protocols developed for this work. Female C57BL/6 mice (Charles River) were used for infections. Inoculum was administered orally at a ratio of 1:1 of wild type *S.* Typhimurium harbouring a chloramphenicol cassette and a non-polar *ssaN* mutant strain as described previously (Cooper et al., 2011). After 72 hours, spleen liver, and cecum were removed, homogenized, and plated to determine total colony forming units (CFU), then replica plated on chloramphenicol plates to determine total wildtype CFU. Competitive Index (CI) was calculated as CFU (mutant/wild type)$_{output}$/(mutant/wild type)$_{input}$.

Secretion assays

Strains were cultured in 6 ml volumes as described above to an OD_{600} of approximately 0.6 and centrifuged at 10000 *g* for two minutes. The resulting supernatant was isolated as the secreted protein fraction. Secreted protein was then filtered through a 0.2μm filter (Millipore) to remove trace bacteria, and precipitated in a final concentration of 10% trichloroacetic acid at 4°C for 16 hours. Secreted protein was then centrifuged at 16000 *g* for 45 minutes and washed with acetone before re-suspending in 2x sample buffer (1 M Tris pH 8.0, 20% SDS, 0.5 M EDTA pH 8, 10% glycerol, 200 mM dithiothreitol) according to culture OD_{600}. Cytoplasmic protein was also re-suspended according to culture OD_{600}. Standard western blotting techniques were used with antibodies directed to HA (1:2000 in 5% skim milk powder in TBST), and DnaK (1:5000). Secondary antibodies (1:5000) were conjugated to horse radish peroxidase, detected with enhanced chemiluminescence, and exposed to Hyperfilm (GE).

Protein production and purification

SsaN protein was produced and purified as described previously (Cooper et al., 2010). Briefly, *E. coli* strain DE3 was grown with *ssaN*Δ1-89 cloned in a pET3A vector with a C-terminal HIS tag. Cells were lysed, and subjected to nickel and ion-exchange column chromatography at 4°C.

Crystallization, data collection and structure determination

Crystals of SsaN were grown at 20°C using the hanging-drop vapour diffusion method. A volume of 2 μL of SsaN (1.7 mg/mL in 0.02 M Tris pH 7.5, 0.1 M potassium chloride, and 0.01 M TCEP) was mixed with 1μL of crystallization solution (0.5 M ammonium sulfate, 0.1M sodium citrate tribasic dihydrate pH 5.6, and 10% v/v Jeffamine M-600) and 0.2uL of 0.1M L-

proline. The drops were initially dehydrated against 500uL of 1.5M ammonium sulfate. Following nucleation, the drops were transferred over successively higher concentrations of well solution to a final concentration of 4M ammonium sulfate. Diffraction data was collected at a wavelength of 1.1Å on the X25 beamline of the National Synchrotron Light Source at Brookhaven National Laboratory. The data was processed and scaled with HKL2000 to 2.1Å. A search model was generated from the structure of EscN from EPEC (PDB 2OBM) using the chainsaw algorithm (Winn et al., 2011), which was used to solve the structure of SsaN by molecular replacement using PHASER from the PHENIX software package (Adams et al., 2002). Model building and refinement was carried out through multiple iterations of Coot (Emsley & Cowtan, 2004) and PHENIX-Refine (Adams et al., 2002) until R values and geometry statistics reached suitable ranges (Table 4.1).

Membrane localization assays

Strains were cultured in 150 ml volumes as described above to an OD_{600} of approximately 0.6 and centrifuged at 3000 *g* for 15 minutes. Bacterial pellets were then re-suspended in PBS containing one tablet of protease inhibitor tablets per 50ml (PBS-PI) (Roche). Suspensions were then sonicated for 30 seconds at 60 second intervals six times on ice (power setting 2, Misonix Sonicator 3000), and clarified by centrifugation at 8800 *g* for 40 minutes at 4°C. Supernatants were centrifuged at 200000 *g* for 1 hour at 4°C in an Optima MAX-E ultracentrifuge (Beckman). The pellet formed was re-suspended in PBS-PI mixed with 2x SDS sample buffer and subjected to standard western blotting techniques using antibodies directed at YidC (1:10000), FLAG (1:5000), and DnaK (1:5000).

Results

Loss of the SPI-2 encoded ATPase SsaN reduces the secretion for the SPI-2 associated effector SseL

To ensure that SsaN is required for T3SS function, the secretion of the SPI-2 associated effector SseL was examined in an *ssaN* deletion strain and an *ssaN* complementation strain. While levels of SseL remain constant in cytoplasmic fractions, secreted SseL levels are decreased but not abolished in the *ssaN* mutant strain compared to the complimented strain. This suggests that either SsaN is not absolutely critical for secretion or that SseL may also be secreted in a non-SPI-2 dependent manner. Normal secretion is restored in the complemented strain suggesting a lack of pleiotropic effects from the chromosomal manipulation (Figure 4.1).

ssaN is required for fitness in a mammalian infection model

The fitness defect caused by an *ssaN* mutant strain was examined during a competitive infection in mice. A wild type strain harbouring a chloramphenicol resistance cassette was directly competed against an *ssaN* deletion strain at a ratio of 1:1. After 72 hours, spleen, liver, and cecum were removed homogenized and plated to determine wild type and *ssaN* mutant strain survival. A CI value of less than 0.1 was found for all data points in the liver and spleen, while an average value of 0.2 was found in the cecum (Figure 4.2). This suggests that SsaN is required for *S.* Typhimurium replication within a mammalian host.

The structure of SsaN

The structure of SsaN with an 89 amino acid N-terminal truncation, required to produced soluble protein, was solved by molecular replacement with the orthologous T3SS ATPase EscN found in

EPEC (Zarivach et al., 2007). SsaN consists of twelve α-helices wrapped around an inner seven stranded parallel β-sheet which acts as a platform for large C-terminal and N-terminal motifs each consisting of four α-helices (Figure 4.3). When modelled into a hexamer conformation, an inner channel is formed which is lined by three internal regions from each monomer (Figure 4.4). The region which projects most internally in the central channel extends from β-8 suggesting a potential surface mediating effector secretion. Additional exposed channel surfaces are at the end of β-4 and β-9 which exist as coil and an α-helix conformation respectively. The two Walker boxes are in conserved regions for both EscN and SsaN, Walker A at the end of α-2, and Walker B within β-9. Also in conserved regions, the arginine finger motif at R352 of SsaN is along an extended region of coil between α-9 and α-10, which contributes to ATP binding and possibly hexamer formation. Interactions between virulence chaperones and the T3SS ATPase have been shown in the past, however these interactions have yet to be mapped on the ATPase itself (Akeda & Galan, 2005, Thomas et al., 2005, Thomas et al., 2007). Potential regions where this interaction may occur on SsaN include hydrophobic patches V273-P279 and L379-I383 at the C-terminus that adopt alternate conformations compared to EscN. These differences in conformation between SsaN and EscN could be potential protein interaction sites, as different virulence chaperones have been shown to interact with these proteins. At V273-P279, SsaN has a disordered coil that connects α-6 and α-7, this region in EscN contains a longer α-6 that comes close to contacting α-7. The residues at L379-I383 of SsaN are in α-10, this region is in a coil between α-10 and α-11in EscN, resulting in an alternate conformation between these two proteins. The structures of EscN and SsaN differ slightly in the size of the channel opening at the N-terminus, with SsaN having a more opened conformation than EscN (33Å / 30 Å). Conversely, at the C-terminus, EscN has a much more narrow or sheltered opening to the pore

than SsaN (18Å / 27Å), however this representation does not include the N-terminal globular domain only successfully crystallized in the flagellar system with FliI (Imada et al., 2007). In the FliI structure, the N-terminal domain forms a β-barrel structure that sits on top of a larger flat face of the remaining N-terminus of the structure, close to the pore, however not overlapping the inner N-terminal α-helices shared with SsaN or EscN. This suggests that this N-terminal domain does not contribute to the channel opening at the N-terminus of FliI, as it does not reduce the diameter of the channel in the hexamer model.

The contribution of the inner pore region of the SsaN hexamer model to effector secretion

The inner channel of the T3SS ATPase hexamer model is a candidate pathway for effector passage and secretion. As many characterized apparatus proteins form ring structures similar to the hexamer model of SsaN, with a similar channel diameter, it is conceivable that effectors may use SsaN as an initial point of interaction, unfolding, and entry at the T3SS. In order to probe residues that may be critical in allowing this process, three regions which protrude into the pore of the hexamer model were chosen for mutational analysis. A complete list of point mutations made for SsaN is listed in table 4.2. The hydrophilic DMNE amino acid sequence at position 312 was mutated to RMNR and AMNA. Both mutations were permissive in effector secretion, as compared to the wild type complementation strain. The second channel region was mutated P164R, which did not affect effector secretion, however mutation of the third channel region, R334W, resulted in a decrease of effector secretion similar to the absence of *ssaN* (Figure 4.5).

Membrane localization of SsaN is independent of the N-terminal domain

In order to determine how SsaN interacts with the T3SS, a truncation mutation strategy was used to assay SsaN membrane localization. Truncations of 10, 20, 24, 64, and 89 amino acids were made to assess if specific regions of charged amino acids such as RWGR20 are responsible for membrane localization, suggesting T3SS interaction. After membrane isolation and probing for the presence of SsaN, all expressed truncations localized with the membrane and secreted effectors (Figure 4.6). It should be noted that the fourth truncation (1-64) is not expressed within the cytosol, and is therefore not present in the membrane fraction. These results suggest that the N-terminal domain (1-89) is dispensable for membrane localization, and that further regions of SsaN are likely responsible for protein interaction with the T3SS.

Discussion

The role of T3SS ATPases is to enable effector secretion by chaperone-effector complex docking, ATP dependent complex dissociation, and effector unfolding (Akeda & Galan, 2005, Gauthier & Finlay, 2003, Gauthier et al., 2003). The inability to secrete effectors in the absence of ATPases leads to a fitness defect during infection common amongst apparatus gene mutations in T3SSs. Here we show that a deletion of *ssaN* reduces effector secretion, and that the structure of SsaN resembles that of EscN, with variations that may shed light on T3SS ATPase function. In addition, the central pore of the hexamer model of SsaN may require charged residues for effector passage as mutation in this region from arginine to proline causes a decrease in effector secretion. Furthermore, a predicted β-barrel N-terminal domain thought to be involved in protein interaction is not required for membrane localization of SsaN or secretion of effectors.

The C-terminal region of EscN has been shown necessary for proper effector secretion, specifically at position V393 (Zarivach et al., 2007). This region on α-10 of SsaN has a markedly different conformation in that the helix loop helix found in EscN is replaced by a single longer helix in SsaN. If as suggested, this region is required for chaperone-effector docking, then it is possible that a different conformation is necessary in *Salmonella* possibly to dock with a different set of effectors and chaperones.

A structural comparison between regions of the F_1F_0 α and β subunits and SsaN shows that residues likely to interact with the γ stalk subunit of the F_1F_0 are generally hydrophobic, while these regions are charged and in different arrangements in SsaN. In an attempt to understand how effector proteins interact within the channel of the proposed hexamer model of SsaN, and perhaps how T3SS ATPases evolved from the F_0F_1 protein complex, regions within the central channel of the SsaN hexamer model were mutated in order to determine if charged residues play a role in effector secretion. The first region to be mutated and tested for secretion competence was the DMNE sequence at the end of α-8 at amino acid 312. Upon mutating both the aspartic acid and glutamic acid residues to either arginine or alanine, no difference was seen in secretion competence compared to a wildtype complementation. This led us to choose additional regions for mutational analysis, proline 164, adjacent to α-8 was mutated to arginine and arginine 334 on α-9 was mutated to a tryptophan. The tryptophan mutation resulted in decreased secretion of the effector SseL, indicating that the combination of charged amino acids throughout the channel may be responsible for effector secretion.

The localization of T3SS ATPases has been both directly visualized with tagged GFP proteins, and by probing for protein association with membrane fractions of cellular lysates (Diepold et al., 2010, Andrade et al., 2007). While one study has shown that a specific valine

residue in the N-terminus of the SPI-1 encoded T3SS ATPase InvC can be mutated to inhibit membrane localization (Akeda & Galan, 2004), we attempted to determine what region of the N-terminus, if any, may be responsible for membrane localization of SsaN. We found that all truncations made in the N-terminus, up to and including the complete removal of the predicted β-barrel domain, were found in the membrane fraction. Of note the fourth truncation made was not expressed in the cytosol. This region encompasses the previously identified V51 residue of InvC characterized as contributing to membrane localization. This suggests that either protein interactions between the T3SS ATPase at the T3SS are complex, encompassing more of the body of the protein, or that C-terminal regions may be responsible for interaction.

Recently, EscN has been shown to interact with the "c-ring" protein EscQ at the cytoplasmic face of the T3SS encoded in EPEC (Biemans-Oldehinkel et al., 2011). Moreover, the presence of these proteins were required for effector secretion. Although this study shows that EscN and EscQ interact, there is still no information available for the precise orientation of a T3SS ATPase with the T3SS. Future work is required to determine additional protein interaction sites in SsaN, and the mechanism it uses for effector secretion.

Table 4.1 – Crystallographic data and refinement statistics

Data collection		**Model and refinement**	
Space group	C2	Resolution (Å)[a]	37.03 – 2.10
Cell parameters		R_{work}/R_{free} (%)	18.11/23.04
a,b,c (Å)	138.13, 76.31, 39.09	$Reflections_{observed}$	22,862
α, β, γ (°)	90, 103.701, 90	$Reflections_{Rfree}$	2,015
Molecules in ASU	1	No. atoms	
Resolution (Å)[a]	50.0 - 2.10	Protein	2,551
Unique reflections	22,889	Ligand/ion	0
Redundancy[a]	3.2 (3.2)	Water	125
Completeness (%)[a]	98.1 (98.5)	R.m.s.d. bond	
I/σ(I)[a]	11.1 (2.60)	Lengths (Å)	0.008
R_{merge} (%)[a]	9.7 (51.4)	Angles (°)	1.212
Wilson B Factor (Å^2)	29.03	Average B Factor (Å^2)	48.13

[a] Statistics for the highest resolution shell are shown in parentheses.

Table 4.2 - List of SsaN point mutations generated.

Strain Identifier	Region	Amino Acid Substitution
27-24	Predicted Hexamer Channel 1	DMNE312AMNA
27-25	Predicted Hexamer Channel 1	DMNE312RMNR
27-65	Predicted Hexamer Channel 3	P164R
27-66	Predicted Hexamer Channel 2	R334W
27-28	N-Terminal Domain	Δ1-10
27-29	N-Terminal Domain	Δ1-20
27-30	N-Terminal Domain	Δ1-24
27-31	N-Terminal Domain	Δ1-64
27-49	N-Terminal Domain	Δ1-89

Figure 4.1 –Loss of SsaN reduces SseL secretion. Mutant and complemented *ssaN* strains were grown in SPI-2 inducing conditions and centrifuged. Supernatants were collected, filtered, and concentrated as secreted protein fractions. Both cytosolic and secreted fractions were probed with standard western blotting techniques. DnaK was used as a loading control.

Figure 4.1

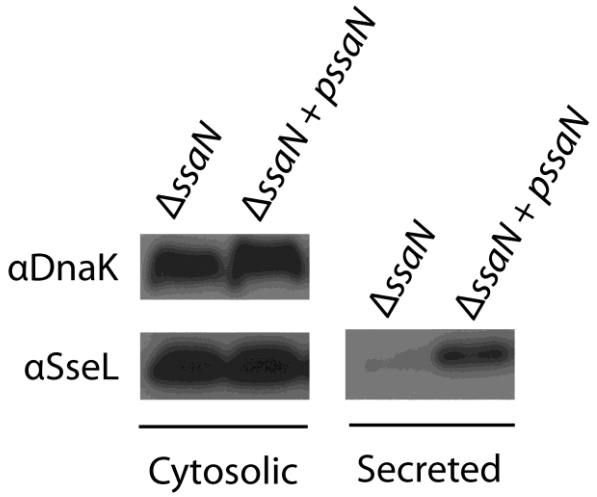

ΔssaN
ΔssaN + pssaN
ΔssaN
ΔssaN + pssaN
αDnaK
αSseL
Cytosolic
Secreted

Figure 4.2 – Contribution of *ssaN* to fitness during mammalian infection. Competitive infections (CI) were performed in female C57BL/6 mice by oral inoculation of equal amounts of marked wild type and mutant strain. After 72 indicated organs were dissected, and plated to give the above CI values of wild type to *ssaN* mutant.

Figure 4.2

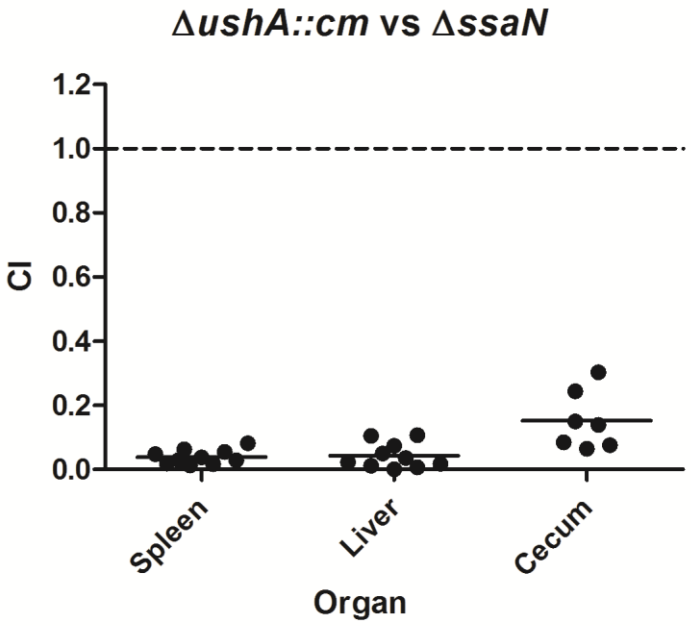

ΔushA::cm vs ΔssaN
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CI
Spleen
Liver
Cecum
Organ

Figure 4.3 – Monomeric structure of SsaN. Three regions protruding into the central channel are shown in red (D312), blue (P164), and magenta (R334). (A) Structure is shown from N-terminus (Top) (N) to C-terminus (Bottom) (C). (B) Structure shown from top view, 90 degree forward tilt from A.

Figure 4.3

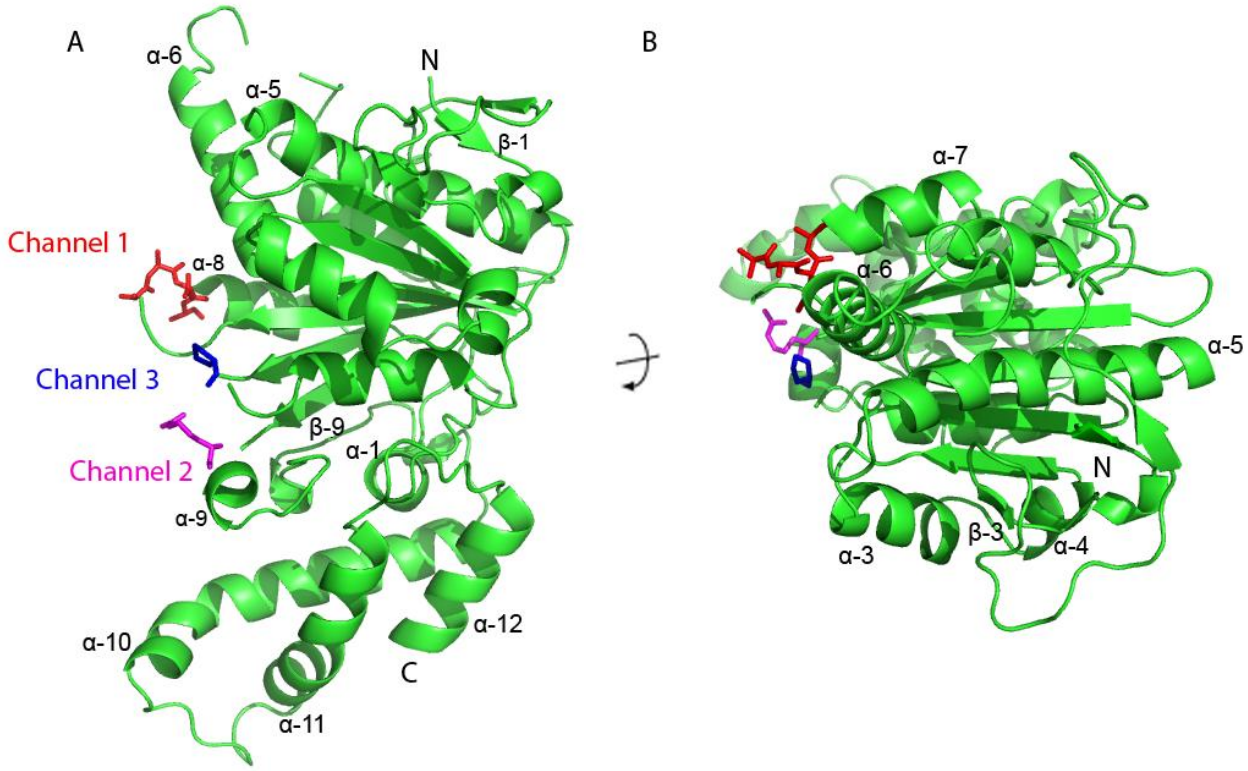

A
α-6
N
α-5
β-1
Channel 1
α-8
Channel 3
β-9
α-1
Channel 2
α-9
α-10
α-12
C
α-11
B
α-7
α-6
α-5
N
β-3
α-4
α-3

Figure 4.4 – SsaN modelled as a hexamer. (A) from N-terminus (top) to C-terminus (bottom). (B) From an overhead view of (A). Each monomer represented by a different colour.

Figure 4.4

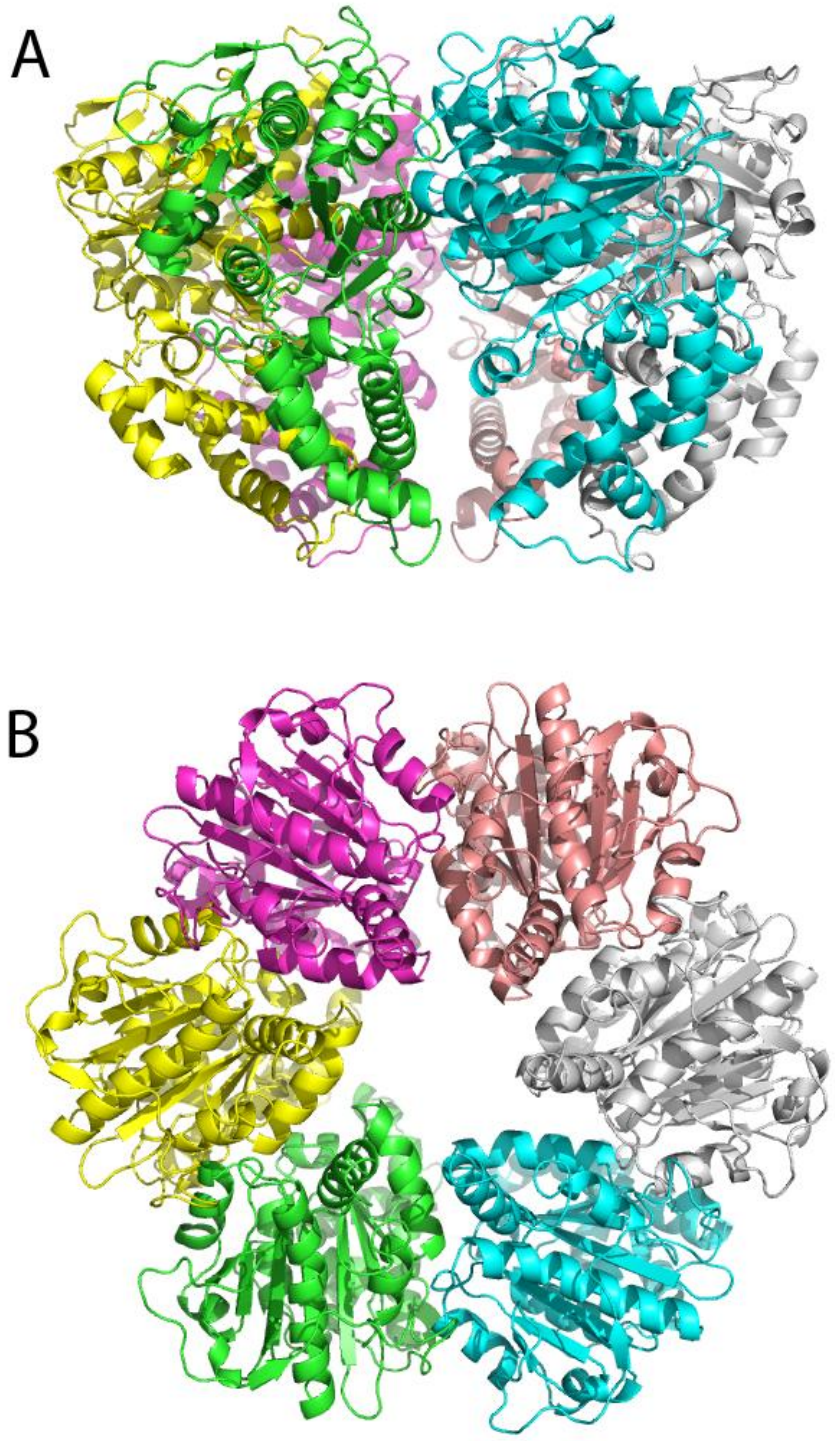


Figure 4.5 – The central channel of SsaN requires negatively charged residues for secretion of SseL. SseL protein was probed in the secreted fraction of indicated strains grown in SPI-2 inducing conditions. Channel mutants are labelled pCH1 (DMNE312AMNA), pCH2 (DMNE312RMNR), pCH3 (R334W), and pCH4 (P164R).

Figure 4.5

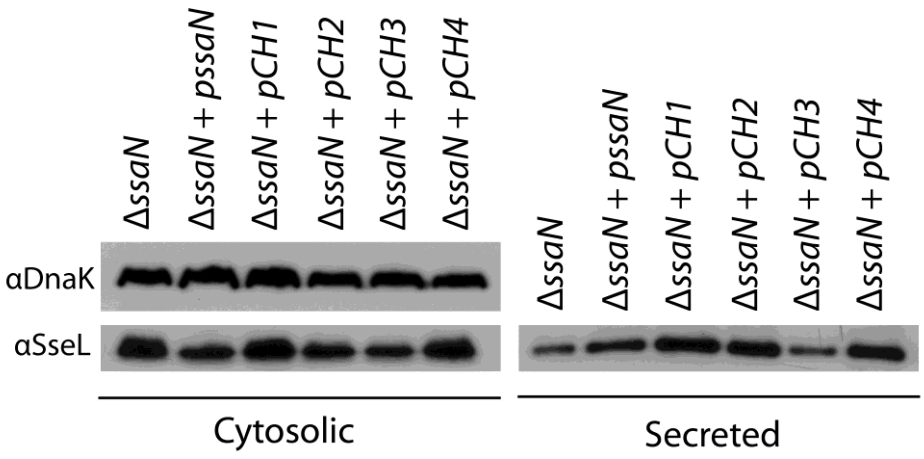
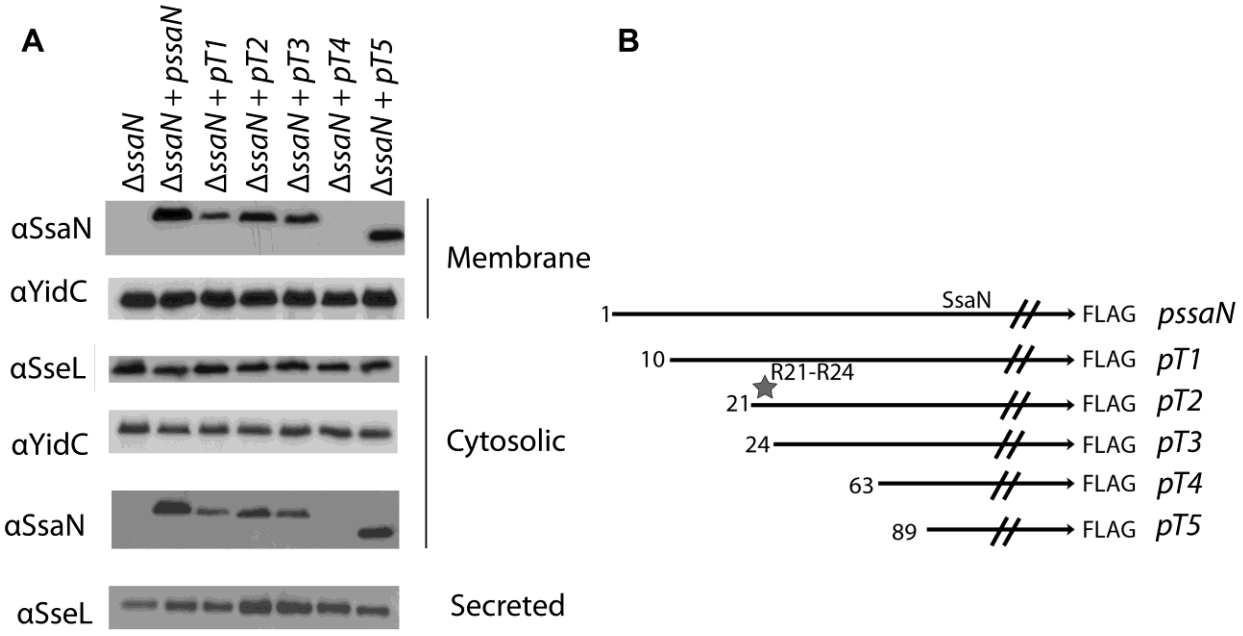

ΔssaN
ΔssaN + pssaN
ΔssaN + pCH1
ΔssaN + pCH2
ΔssaN + pCH3
ΔssaN + pCH4
αDnaK
αSseL
Cytosolic
ΔssaN
ΔssaN + pssaN
ΔssaN + pCH1
ΔssaN + pCH2
ΔssaN + pCH3
ΔssaN + pCH4
Secreted

Figure 4.6 – N-terminal truncations of SsaN localize to the membrane. (A) Indicated strains were grown in SPI-2 inducing conditions, pelleted, sonicated and centrifuged to isolate membrane or secreted fractions. YidC was used as a positive membrane control. No DnaK was found in either the secreted or membrane fraction (data not shown). Truncations are labelled T1 (Δ1-10), T2 (Δ 1-20), T3 (Δ 1-24), T4 (Δ 1-64), and T5 (Δ 1-89). (B) Diagram of *ssaN* constructs depicting indicated truncations.

Figure 4.6


A
ΔssaN
ΔssaN + pssaN
ΔssaN + pT1
ΔssaN + pT2
ΔssaN + pT3
ΔssaN + pT4
ΔssaN + pT5
αSsaN
αYidC
Membrane
αSseL
αYidC
αSsaN
Cytosolic
αSseL
Secreted
B
SsaN
FLAG
pssaN
pT1
pT2
pT3
pT4
pT5
R21-R24
1
10
21
24
63
89

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Chapter V – Discussion

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Opening statements

Virulence strategies of bacterial pathogens provide remarkable examples of the evolutionary process microbes use to gain a fitness advantage. The horizontal acquisition and regulation of T3SSs and their satellite proteins, effectors, and virulence chaperones are excellent examples of these processes. The identification and functional characterization of these proteins aids in the clarification of how these pathogens have evolved and how they increase fitness by usurping and utilizing host metabolic pathways and niches. The past three chapters describe the characterization of proteins essential to the virulence of *Salmonella enterica*, greatly expanding our knowledge of *Salmonella* pathogenesis.

The identification and functional characterization of SrcA provides the first example of a multi-cargo virulence chaperone for SPI-2 associated effectors. The SrcA cargo, consisting of PipB2 and SseL, was also identified and a mechanism necessary for their secretion characterized. Additionally, a new chaperone substrate identification protocol has been developed which may aid in locating cargo for other virulence chaperones. Furthermore, the structural characterization of SrcA reveals that a conserved structural design exists for a virulence chaperone associated with the SPI-2 T3SS.

The SPI-2 encoded translocon component SseC had no previously described virulence chaperone, the only distal T3SS component lacking this designation. SseC, along with its virulence chaperone SscA, are essential for virulence, and required for regulation of SPI-2 promoters. This finding highlights the importance of this work that yet another layer in SPI-2 regulation has been elucidated.

Characterization of both the motif properties and the structural composition of the SPI-2 encoded T3SS ATPase SsaN is a key finding in the understanding of *Salmonella* pathogenesis. We have demonstrated that this essential enzyme has an N-terminal domain that is dispensable for membrane localization and effector secretion and that a specific composition of residues within the predicted hexamer channel may be required for function. The structure of SsaN resembles the EscN T3SS ATPase encoded within the LEE of EPEC, however several differences in the position of specific α-helices may highlight differences in protein interaction between the two proteins.

Taken together, the identification and characterization of two novel virulence chaperones and an essential apparatus component associated with the SPI-2 encoded T3SS represents a significant advancement in understanding the network of proteins used to enhance intracellular survival of *S. enterica*. The contributions of virulence chaperones are often critical to T3SS function, and are therefore essential to bacterial pathogenesis. These findings also may form the foundation for further advancements, as many questions remain unanswered for the precise regulation, assembly and secretion of substrates for the SPI-2 encoded T3SS.

Remaining questions regarding the SrcA virulence chaperone

The genetic location of *srcA* is interesting, as it is the only known class IB virulence chaperone not found encoded with a corresponding T3SS PAI. The acquisition of *srcA* likely occurred in a horizontal transfer event of a small pathogenicity islet with *sseK2* and *STM2139*. While *sseK2* encodes a secreted effector (Kujat Choy et al., 2004), SrcA does not seem to have a detectable impact on its secretion. *STM2139* does not have any detectable conserved domains nor does it possess any strong sequence similarities with known proteins. It shares weak sequence

similarities with membrane proteins associated with the flagellar system of the eukaryotic single celled green alga *Chlamydomonas reinhardtii* (Wargo et al., 2005). Despite the considerable differences between eukaryotic and prokaryotic flagella, they both contain membrane embedded protein complexes used as a base for extracellular components. Additionally, *STM2139* has polycistronic coding with SrcA, and taken together, this yields the attractive hypothesis that it may be involved in T3SS function.

Additional questions are raised when examining the SrcA substrates PipB2 and SseL. The dynamics of temporal effector delivery have not been addressed for effectors secreted through the same T3SS. The impact that a multi-cargo virulence chaperone may have on the delivery of its individual effector substrates to the T3SS may have a role in determining the dynamics of host cell modulation. If two effectors compete for chaperone availability in the bacterial cytosol, then a higher affinity interaction would yield an earlier delivery to the host cell. This would then presumably result in earlier effector function in the target cell. PipB2 function is required at latter stages of infection. Although we do not know the precise kinetic requirements of SseL during infection, one can speculate that SseL outcompetes PipB2 at earlier infection time points. Further studies would be required to measure temporal effector deliver and binding affinities of these proteins. If this model is verified, it would be possible that virulence chaperones regulate protein secretion at the T3SS, with the consequences of temporal regulation of effector function in the host cell.

Further implications for the SscA-SseC complex in regulation

The interaction between the class II virulence chaperone SscA and its translocon substrate SseC is significant for multiple reasons. The canonical property that SscA aids in the secretion of

SseC is apparent, however a role for this interaction in gene regulation must also be considered. As both SseC and SscA affect the regulation of SPI-2 promoters, and these proteins interact with one another, the SscA-SseC complex formation may dictate their individual regulatory roles. Additionally, the mechanism for SseC gene regulation properties is interesting, as it is a molecule that likely polymerizes in an oligomeric ring structure, supported by a separate protein ring (SseD) and a translocon platform protein (SseB), and is found in the eukaryotic membrane and therefore would likely not have a dual role as a transcription factor. This may suggest that its presence in the bacterial cell is part of a feedback loop, interacting with other transcriptional protein complexes.

An additional factor to consider is a role for the SscA-SseC complex in binding the sorting platform composed of SsaBLM. As discussed previously, this sorting platform is a pH dependent protein complex that regulates the secretion of translocon components and effectors (Yu et al. 2010). Whether or not an interaction between SscA-SseC and the SsaBLM complex occurs remains to be determined, however if this interaction occurs as suggested by the authors describing the sorting platform, this would likely affect other aspects of the SscA-SseC complex function (Figure 5.1).

Implications of SsaN as a gateway for effectors and a crucial piece of the T3SS cytoplasmic face

Despite several studies characterizing biochemical and structural properties of T3SS ATPases, much is left unknown. These integral pieces of the T3SS are the only proteins with ATP hydrolysis capabilities, and were therefore originally believed to be the power centres for T3SS function (Eichelberg et al., 1994). Complementary to this idea, T3SS ATPases were noted for their amino acid sequence conservation with F_1F_0 ATP synthase subunits, indicating that they

may utilize ATP hydrolysis for secretion. The mutation of residues in the inner channel of the predicted hexamer model may address aspects of T3SS ATPase evolution. The hydrophobic patches found in F_1F_0 α and β subunits for interaction with the γ stalk could possibly inhibit effector secretion had they not evolved to negatively charged residues, and adopted a new conformation. Additional evolutionary steps undergone to convert the F_1F_0 ATP synthase to a T3SS chaperone-effector uncoupling mechanism would be complex, although examples of such conversions have been observed in other systems. The bacterial T6SS has incorporated inverted bacteriophage tail machinery and an ATPase to secrete Hcp and VgrG proteins into eukaryotic cell cytoplasm (Leiman et al., 2009, Jani & Cotter, 2011).

An additional system where structural similarities are mirrored in SsaN is the ClpB family of protease docking proteins. Although much larger than T3SS ATPases, ClpB proteins form hexamers, linked together by arginine finger motifs where two monomers interact with one another to bind ATP using a conserved arginine and glutamine, as seen with the *Thermus thermophilus* structure (Lee et al., 2003) (Figure 5.2). Not only is this structural similarity shared, but so too is the proposed model of protein unfolding and passage through a central pore (Lee et al., 2003). While proteins are often targeted for degradation and passed through ClpB to be degraded by an interacting proteolytic component such as ClpP (ClpBP), effector proteins may pass through T3SS ATPases, and the remaining T3SS apparatus proteins toward the host cytoplasm. The unfolding that takes place at ClpB is ATP dependent precisely akin to the unfolding of effectors and dissociation of virulence chaperones (Akeda & Galan, 2005, Martin et al., 2005).

The N-terminal predicted β-barrel domain of the T3SS ATPase remains an interesting protein domain of unknown function. Experiments described in Chapter IV suggest that it

appears to be dispensable for membrane localization, and therefore perhaps for association with the T3SS apparatus. Additionally, previous studies identified a Valine (V51) residue in InvC responsible for membrane localization (Akeda & Galan, 2004). Interestingly, a truncation mutant spanning the homologous region of SsaN was not found in the cytosolic or membrane fraction, suggesting that mutation of residues in this area may result in unstable proteins. If this region is not required for T3SS apparatus interaction and therefore membrane localization, then its role remains elusive. As it is not present in studies conducted with EscN (Zarivach et al., 2007), or those outlined in chapter II, it would then appear dispensable for ATPase activity and virulence chaperone interaction. Furthermore, no additional N-terminal domain residues were implicated in any function during an InvC mutational analysis (Akeda & Galan, 2004). The only description of function for this domain in the related FliI protein is interaction with the apparatus protein FliH (Minamino & MacNab, 2000), and the function of this domain in the F_1F_0 complex is protein interaction with the δ subunit (Wilkens et al., 2005). Both FliH and the F_1F_0 complex δ subunit share no sequence or structural similarity to T3SS proteins. Further analysis, perhaps using a more broad investigation such as the domain's contribution to fitness in a mammalian infection model, may provide insight as to the necessity of this region.

The significance of two T3SS in S. enterica

As *S. enterica* diverged from *S. bongori*, it acquired a second T3SS encoded on SPI-2. The metabolic cost for possessing, regulating, and assembling two T3SSs is presumably higher than the acquisition and integration of additional effectors into the SPI-1 T3SS secretion repertoire. This asks the question of how *S. enterica* maintains two T3SS, for the secretion of over 30 effectors instead of using one. Several aspects may have lead to the stable integration of the

second T3SS. The regulation of SPI-2 is complex, with multiple positive and negative inputs. These inputs are linked to environmental factors which likely act to initiate T3SS assembly. Only a subset of these inputs overlap for SPI-1 regulation, perhaps highlighting the acquisition and integration processes for the second T3SS rather than a complete rewiring of regulatory pathways to include SPI-1 expression in traditional SPI-2 inducing environments. Furthermore, a fitness advantage for the acquisition of SPI-2 must be considered. Certain effectors have been shown to be secreted through either the SPI-1 or SPI-2 encoded T3SS but not both. Effectors with temporally distinct functions include SipA used for actin remodelling during initial invasion, and SifA used for SCV maintenance after internalization has occurred. Given that these effectors have distinct functions at given time points during infection, dual T3SS may aid in this differential temporal delivery.

Since the initial characterization of SPI-2, the view of a specific T3SS for epithelial cell invasion (SPI-1) and a specific T3SS for macrophage specific intracellular survival (SPI-2) has dominated. However, this theory has been re-evaluated due to several observations including: the presence of effectors secreted through both the SPI-1 and SPI-2 encoded T3SSs, cross-transcriptional regulation of each PAI, and the discovery of effectors with dual functions. The secretion of SspH1, GogB, and SlrP has been documented as occurring through either the SPI-1 or SPI-2 encoded T3SS (Coombes et al., 2005, Tsolis et al., 1999, Haraga & Miller, 2003). Whether these effectors have evolved to take advantage of dual secretory pathways, are able to recognize the cytoplasmic face of either T3SS due to their unique structure, or have unidentified virulence chaperones that enable this secretion is not clear. The acquisition of these dual secretion pathways provides evidence that definitive functions for each T3SS are not as easily categorized.

Connections between transcriptional regulation of both SPI-1 and SPI-2 are also becoming apparent. Evidence suggests that the regulators FIS and IHF both up-regulate promoters in SPI-1 and SPI-2, and the SPI-1encoded regulator HilD up-regulates the *ssrA* promoter in SPI-2 (Ellermeier & Slauch, 2007). Additionally, recent data supports a role for an anti-sense RNA transcribed within the *hilA* regulatory gene encoded in SPI-1. The transcription of this RNA was identified as a binding site for the SPI-2 encoded regulator SsrB (Tomljenovic-Berube et al., 2011) (Osborne, unpublished data). As common regulatory mechanisms between the two SPIs exist, this may allude to overlapping functions. Furthermore, the role of effectors with dual functions such as SptP (Humphreys et al., 2009), further support the idea that the two T3SS of *S. enterica* have more common function than originally thought. It is an interesting evolutionary path that *S. enterica* has undergone to distinguish itself from the ancestral *S. bongori*. Fascinating discoveries have characterized the functional integration of the SPI-2 encoded T3SS, its virulence chaperones, and effectors.

Activation of T3SSs

The activation of T3SSs is an often complex and tightly regulated process, as the assembly of the apparatus and secretion of effectors is likely metabolically costly. Although various transcription factors have been identified that have known environmental cues for their activation of T3SS related genes across several species, little is known of the cues for secretion of effectors. One model of secretion activation involves proteins localized to the cytoplasmic face of the T3SS. Proteins such as the YopN-TyeA and SsaBLM complexes are thought to physically inhibit or allow secretion of different secreted substrates. Both complexes are required for translocon complex protein secretion and inhibition of effector secretion at early infection time

points (Yu et al., 2010, Coombes et al., 2004, Sundberg & Forsberg, 2003, Schubot et al., 2005). However, the mechanism by which these sorting platform complexes respond is unknown. An additional model for T3SS activation involves conformational changes within the needle filament itself. Mutations in needle filament monomers have been examined that lead to different secretion phenotypes. In *Yersinia*, mutations in the needle filament YscF constitutively secrete Yops in the presence or absence of calcium (a *Yersinia* T3SS activating agent), prior to contact with a eukaryotic cell, suggesting that the needle filament is in some way involved in secretion regulation (Torruellas et al., 2005). A similar study was conducted for the needle filament protein MxiH of *Shigella*. This study found residues in MxiH which were required for the regulation of secretion, and for this secretion to be induced by low calcium concentrations, further indicating a role for the needle filament in effector secretion regulation (Kenjale et al., 2005). While environmental signals and proteins involved in T3SS activation are known, additional research is needed to highlight the sensor cascades present to sense these environmental signals and direct the identified proteins to enact T3SS function.

Inhibition of T3SSs

Due to its role in virulence in a wide variety of pathogens, treatments that could inhibit T3SS function would represent a major advancement in the treatment of pathogens harbouring a T3SS. In light of this, several small molecule screens, and protein interaction studies have been undertaken to come up with clinically relevant treatments for pathogens. Utilizing high-throughput screening, a study conducted in *Yersinia* identified compounds that would inhibit the ATPase activity of the T3SS ATPase YscN, which in turn showed a loss of YopE secretion, and attenuation in cell culture models (Swietnicki et al., 2011). An additional study investigated

small molecules that inhibited T3SS gene expression in enterohemorrhagic *E. coli* (EHEC). Compounds known as salicylidene acylhydrazides decreased gene expression within the LEE (Tree et al., 2009). Another study conducted in *Yersinia* showed a decrease in Yop secretion in response to a chemical identified in a small molecule screen, this compound was also shown to inhibit secretion in an epithelial cell culture model of infection (Kauppi et al., 2003). While the identification of these compounds is promising, a multitude of challenges are still faced when developing a therapeutic suitable for mammalian consumption, and to date, none of these treatment options have yet reached the clinic.

Future directions

A multifaceted, systematic approach to the characterization of SrcA is illustrated in chapter II, yet other virulence chaperones likely exist to aid in the secretion of the remaining SPI-2 associated effectors. As these effectors are required for infection, knowledge of their mechanism of secretion is valuable, and should be investigated. A screening method for detecting effector secretion coupled to a mutagenesis protocol, such as signature tagged mutagenesis, would be one approach for identifying virulence chaperones required for the secretion of effectors. Any non-essential gene mutations that are not responsible for T3SS apparatus, but result in loss of effector secretion would be candidate genes for characterization. Additionally, the concept of effector-chaperone docking at the T3SS ATPase should be investigated for chaperone-effector pairs in *S. enterica* specifically at both T3SS ATPases (SsaN and InvC). As previous studies indicate that interspecies secretion of effectors is possible when accompanied by the appropriate chaperone (Rosqvist et al., 1995), this is perhaps an occurrence in *S. enterica* between the two respective

T3SSs. Interaction studies between the T3SS ATPases and effector-chaperone complexes are necessary to examine secretion of effectors through either the SPI-1 or SPI-2 encoded T3SS.

The finding that SseC and SscA have a role in SPI-2 regulation is interesting, however follow up studies are needed to determine the mechanism of action. Protein interaction studies should be utilized to determine the interacting partners of an SscA-SseC complex. It is likely that either or both of these proteins have a transcriptional activation or repression binding partner. Additionally, as SscA is required for the secretion of SseC, an interaction with the SsaBLM sorting platform likely occurs, as this complex is formed during the secretion of translocon components such as SseC (Yu et al., 2010). Expanding on previous studies characterizing this platform structure is necessary to determine a more precise function for T3SS substrate recognition.

The characterization of SsaN is incomplete, however initial findings presented in chapter IV should be the basis for future work. A docking mechanism for SsaN with the T3SS does not require the first 90 N-terminal residues, however several other motifs in the protein may function to do so. A similar truncation mutant study should be conducted for the C-terminus, as well as alanine scanning for outer regions of the proposed hexamer model. This may highlight the mechanism of SsaN interaction with the T3SS. Additionally, a mechanism of chaperone-effector docking with a T3SS ATPase has yet to be elucidated, and should be investigated as it is a critical role for T3SS function and a necessary process for *S. enterica* virulence (Akeda & Galan, 2005). Further crystallography methods, such as using the SrcA-SsaN complex for structure determination, and the identification of other chaperone docking proteins may aid in the determination of a binding mechanism for virulence chaperones at the T3SS ATPase.

The results presented and discussed in this thesis shed light on novel mechanisms used by *S. enterica* during infection of host cells. While key proteins involved in these processes have been identified and characterized, the network of virulence chaperones associated with the SPI-2 encoded T3SS remains to be fully identified. These findings will act as a stepping-stone to the complete characterization of this virulence chaperone network and the precise mechanisms used in the *S. enterica* virulence strategy.

Figure 5.1 – Structural schematic of SPI-2 with and without sorting platform directing secreted substrates. The SPI-2 encoded T3SS forms within the outer (OM) inner (IM) and host cell (HCM) membranes. These are the predicted locations of SPI-2 encoded proteins based on the localization of homologs. (A) The sorting platform (SsaBLM) is shown allowing class II virulence chaperone SscA and translocon cargo SseC to dock with the T3SS, and preventing the binding of the class IA virulence chaperone SscB and effector substrate SseF. (B) Also depicted is the interaction of SscB and SseF with the T3SS in the absence of the sorting platform, as well as SseF shown secreted into the host cell cytoplasm.

Figure 5.1

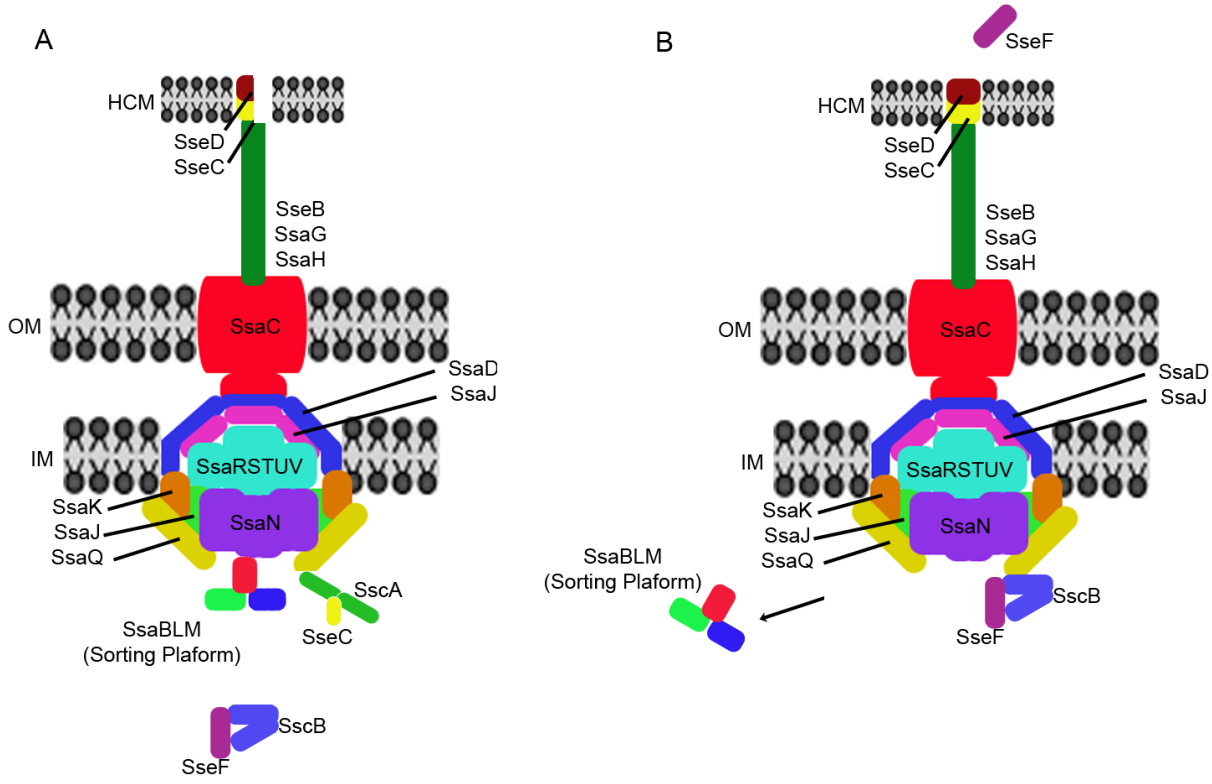
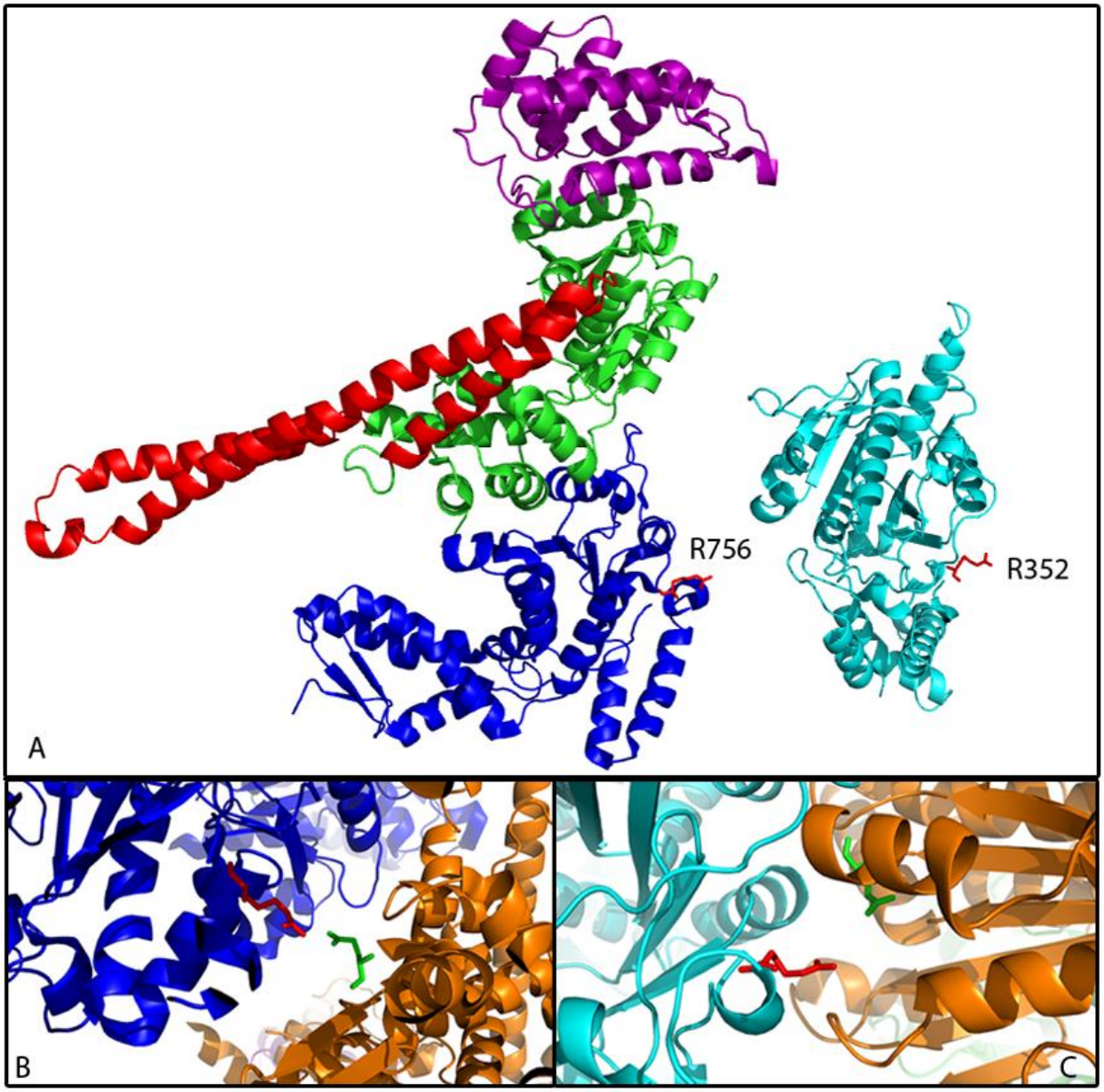

A
HCM
SseD
SseC
SseB
SsaG
SsaH
OM
SsaC
SsaD
SsaJ
IM
SsaRSTUV
SsaK
SsaJ
SsaQ
SsaN
SscA
SseC
SsaBLM
(Sorting Plaform)
SscB
SseF
B
SseF
HCM
SseD
SseC
SseB
SsaG
SsaH
OM
SsaC
SsaD
SsaJ
IM
SsaRSTUV
SsaK
SsaJ
SsaQ
SsaN
SsaBLM
(Sorting Plaform)
SscB
SseF

Figure 5.2 – The arginine finger motif of SsaN resembles a similar motif in ClpB. (A) Both proteins in monomer form, ClpB left, SsaN right with arginine finger motifs highlighted in red. (B) Close up of ClpB monomer blue, interacting with adjacent monomer orange, with arginine finger motif in red and glutamine from adjacent monomer in green. (C) Close up of SsaN monomer cyan, interacting with adjacent monomer orange, with arginine finger motif in red and glutamine from adjacent monomer in green.

Figure 5.2



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