Identification of the Docking Site between a Type III Secretion System ATPase and a Chaperone for Effector Cargo*§

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Background: Chaperones engage type III secretion system (T3SS) ATPases to facilitate effector secretion. Results: The molecular basis of the chaperone-T3SS ATPase interaction interface was elucidated. Conclusion: The C-terminal region of T3SS ATPases mediates binding with multiple contact points along the chaperone. Significance: The chaperone-T3SS ATPase interaction is important for Salmonella pathogenesis and may be a target for anti-virulence drugs.

A number of Gram-negative pathogens utilize type III secretion systems (T3SSs) to inject bacterial effector proteins into the host. An important component of T3SSs is a conserved ATPase that captures chaperone-effector complexes and energizes their dissociation to facilitate effector translocation. To date, there has been limited work characterizing the chaperone-T3SS ATPase interaction despite it being a fundamental aspect of T3SS function. In this study, we present the 2.1 Å resolution crystal structure of the Salmonella enterica SPI-2-encoded ATPase, SsaN. Our structure revealed a local and functionally important novel feature in helix 10 that we used to define the interaction domain relevant to chaperone binding. We modeled the interaction between the multicargo chaperone, SrcA, and SsaN and validated this model using mutagenesis to identify the residues on both the chaperone and ATPase that mediate the interaction. Finally, we quantified the benefit of this molecular interaction on bacterial fitness in vivo using chromosomal exchange of wild-type ssaN with mutants that retain ATPase activity but no longer capture the chaperone. Our findings provide insight into chaperone recognition by T3SS ATPases and demonstrate the importance of the chaperone-T3SS ATPase interaction for the pathogenesis of Salmonella.

Many Gram-negative pathogens employ a specialized secretion complex known as a type III secretion system (T3SS)† for the translocation of bacterial virulence proteins into host cells. These secretion systems, composed of over 20 proteins, span the inner and outer bacterial membranes and cross the eukaryotic cell membrane to form a channel through which largely unfolded effectors pass (1). Effectors, in complex with specific secretion chaperones, are targeted to the base of T3SSs. Here, a highly conserved ATPase aids in chaperone release and effector unfolding (2), and energy derived from the proton motive force drives effector secretion (3, 4). Some of the earliest (5, 6) and most recent demonstrations (7) of chaperone-T3SS ATPase interactions have come from work on the enteropathogenic Escherichia coli T3SS ATPase, EscN, and the chaperones, CesT and CesAB. However, despite a crucial role in the secretion process, little is known about how T3SS ATPases recognize their repertoire of chaperones and effector substrates.

Salmonella enterica serovars, a group of pathogens that cause diseases such as gastroenteritis and typhoid fever (8, 9), employ two distinct virulence-associated T3SSs for the translocation of unique sets of effector proteins into the host (1). The T3SS encoded by Salmonella pathogenicity island 1 (SPI-1) is activated upon contact with intestinal epithelial cells and is required for the invasion of non-phagocytic cells (10). The second T3SS, encoded by SPI-2, translocates effector proteins into the host cell that are necessary for ensuring intracellular survival and replication (11, 12). Chaperone-effector interactions have been charted extensively for both the T3SS-1 (13–16) and T3SS-2 (17–22), and a number of functions attributed to secretion chaperones beyond that of effector binding have been demonstrated in Salmonella (23, 24).

T3SS ATPases share significant structural similarity to the catalytic β subunit of F0F1-ATPases (25, 26) and form hexameric (26) and dodecameric (27) rings that reside at the interface of the cytoplasmic membrane, probably aligning with the base of the T3SS to form a continuous channel (27, 28). Both oligomerization and membrane association enhance the catalytic activity of these enzymes (29, 30). At the membrane, T3SS ATPases interact with chaperones and other components of the export apparatus to ensure that effector substrates are delivered to the secretion channel. Loss-of-function mutations in these ATPases result in severe effector secretion defects and strong
virulence attenuation phenotypes (31–33). One of the most functionally characterized T3SS ATPases is the SPI-1-encoded InvC from S. enterica, for which particular domains involved in catalytic activity, membrane association, and oligomerization have been defined (31). Although recent structural and biochemical work has aided in understanding the role of these T3SS ATPases in effector secretion, the molecular basis for the chaperone-T3SS ATPase interaction has remained elusive.

In this work, we used the S. enterica SPI-2-encoded ATPase, SsaN, as a model for uncovering fundamental chaperone binding functionality, given the large repertoire of chaperones and effectors associated with the T3SS-2. In doing so, we defined the chaperone interaction site on this T3SS ATPase and identified the corresponding residues on the multicargo effector chaperone, SrcA, that facilitate binding to SsaN. The high degree of conservation of the chaperone-binding site among T3SS ATPases suggests that insight into the mechanism of substrate recognition provided here probably extends to virulence-associated type III secretion systems in other species.

**EXPERIMENTAL PROCEDURES**

**Competitive Infections**—All animal experiments were conducted in accordance with the guidelines set by the Canadian Council on Animal Care and were approved by the McMaster University Animal Review Ethics Board. Female C57BL/6 mice (Charles River, Wilmington, MA) were infected via the peritoneum with 2 × 10^9 bacteria in 0.1 mL HEPES, pH 8.0, and 0.9% NaCl containing a 1:1 ratio of chloramphenicol-resistant wild-type S. Typhimurium (ushA::Cm) to an unmarked ssaN mutant, as described previously (34). At 72 h postinfection, the spleen, liver, and cecum were harvested, homogenized in PBS, and plated to determine the total number of colony-forming units (cfu). Colonies were replica-plated onto LB-agar containing either chloramphenicol or streptomycin to determine the total number of wild-type and mutant cfu and the competitive index (cfu). Colonies were replica-plated onto LB-agar. Colonies sensitive to kanamycin and chloramphenicol were selected on medium containing 5% (w/v) sucrose and were then plated onto LB-agar. Colonies sensitive to kanamycin and chloramphenicol were confirmed by sequencing. For chromosomal integrations, the NdeI/KpnI sites of pFLAG-CTC, and the XhoI/KpnI sites of pET3a or pET28b, were cloned into the NdeI/BamHI sites of pCP20. All strains generated were confirmed harboring pFLAG or pET vectors were grown at 37 °C in LB medium with an A_600 of 0.5, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, and grown for an additional 3 h. Cells were harvested by centrifugation (4850 × g for 10 min) and then resuspended in PBS containing a mini-EDTA protease inhibitor tablet (1 tablet/40 mL of PBS) (Roche Applied Science). Cells were lysed by sonication (Misonix Sonicator 3000, Misonix, Farmingdale, NY), and the lysate was spun at 4850 × g for 15 min. The resulting supernatant was filtered through a 0.45-μm filter. Purified SsaN protein was produced and purified as described previously (18). ATPase activity of purified SsaN protein was determined by measuring the release of total phosphate at 37 °C in a microplate assay with BIO-MOL Green according to the manufacturer’s instructions (Enzo Life Sciences). Briefly, purified protein (50 μg/mL) was incubated in reaction buffer (50 mM Tris, pH 7.5, 1 mM MgCl₂, 100 μg/mL BSA, and 1 mM ATP) for 30 min, and then total phosphate release of SsaN variants was measured and normalized to that of wild-type SsaNΔ1–89.

**Protein Purification, Activity Assays, and Circular Dichroism**—SsaN protein was produced and purified as described previously (18). ATPase activity of purified SsaN variants was determined by measuring the release of total phosphate at 37 °C in a microplate assay with BIO-MOL Green according to the manufacturer’s instructions (Enzo Life Sciences). Briefly, purified protein (50 μg/mL) was incubated in reaction buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 100 μg/mL BSA, and 1 mM ATP) for 30 min, and then total phosphate release of SsaN variants was measured and normalized to that of wild-type SsaNΔ1–89.

**Circular dichroism spectra of wild-type SsaNΔ1–89 and variants were collected with an AVIV spectropolarimeter using a quartz cuvette with a path length of 0.1 cm. Purified protein samples were adjusted to concentrations of 0.11–0.17 mg/mL in buffer containing 5 mM HEPES, pH 7.5. Scans were conducted every 1 nm from 198 to 260 nm, and the temperature was maintained at 25 °C. The
Chaperone Recognition by a T3SS ATPase

TABLE 1
Data collection and model refinement statistics

| Parameter                   | Value                  |
|-----------------------------|------------------------|
| Data collection             |                        |
| Space group                 | C2                     |
| Cell parameters             |                        |
| a, b, c (Å)                 | 138.13, 76.31, 39.09   |
| α, β, γ (degrees)           | 90, 103.701, 90        |
| Molecules in asymmetric units | 1                     |
| Resolution (Å)              | 50.0–2.10              |
| Unique reflections          | 22,889                 |
| Redundancy*                 | 3.2 (3.2)              |
| Completeness (%)            | 98.1 (98.5)            |
| I(0)/I(0)                  | 11.1 (2.60)            |
| Rmerge (%)                  | 9.7 (51.4)             |
| Wilson B factor (Å²)        | 29.03                  |
| Model and refinement        |                        |
| Resolution (Å)*             | 37.03–2.10             |
| Rmerge/Rmerge (%)           | 18.11/23.04            |
| Reflections observed        | 22,862                 |
| Reflections Rmerge (%)      | 2.015                  |
| No. of atoms                |                        |
| Protein                     | 2.551                  |
| Ligand/Ion                  | 0                      |
| Water                       | 125                    |
| Root mean square deviation  |                        |
| Bond lengths (Å)            | 0.008                  |
| Angles (degrees)            | 1.212                  |
| Average B-factor (Å²)       | 48.13                  |
| PDB code                    | 4NPH                   |

* Statistics for the highest resolution shell are shown in parentheses.

RESULTS

SsaN Is Essential for Virulence in an Animal Model of Infection and for Secretion of a Subset of SPI-2 Effectors—SsaN is predicted to be the T3SS-2 ATPase based upon its similarity to other characterized T3SS ATPases and upon its enzymatic activity that has been demonstrated in vitro (18, 44). We first confirmed the essential activity of this enzyme for bacterial infection fitness by competing a ΔssaN mutant against wild-type bacteria in C57BL/6 mice. In these experiments, the competitive index for SsaN-deficient cells was less than 0.1 in the spleen, liver, and cecum (Fig. 1A), indicating that the ssaN deletion strain was significantly attenuated during systemic infection.

Given that T3SS ATPases have been shown to be essential for effector secretion (26, 31, 33), we reasoned that the ΔssaN strain was attenuated in vivo due to impaired release of SPI-2 effectors. To investigate this, we tested the secretion of a panel of translocon and effector substrates in a wild-type and ΔssaN background. As expected, SsaN was required for the secretion of needle and translocon components (Fig. 1B) and the effectors encoded within the SPI-2 locus, SseF and SseG (Table 2). We confirmed that SsaN was responsible for this secretion defect by complementing the ΔssaN strain with a wild-type copy of ssaN under the control of the tac promoter. Secretion of the translocon component, SseC, was partially restored in the complemented ΔssaN strain (Fig. 1C).

By contrast to the requirement of SsaN for the secretion of substrates encoded within the SPI-2 locus, SsaN displayed a mixed dispensability pattern for the secretion of effectors encoded outside of the SPI-2 locus. In fact, a subset of effectors was secreted to near wild type levels in the ssaN mutant (Fig. 1, B and D). To determine whether this subset of effectors was being secreted by the T3SS-1 in the absence of a functional T3SS-2, we constructed ΔinvC and ΔssaNΔinvC strains and examined the secretion of PipB, PipB2, SseL, and SopD2 in these backgrounds. Although these effectors could be secreted in the single ΔssaN and ΔinvC mutants, secretion was completely abolished in the ΔssaNΔinvC double mutant (Fig. 1D and Table 2), indicating that a subset of SPI-2 effectors can be targeted to the T3SS-1 in the absence of SsaN.

Crystal Structure of SsaN—We noted in our secretion profiles that all of the substrates encoded within the SPI-2 locus that required SsaN for secretion have known or putative chaperones. Given that T3SS ATPases have been proposed to function as a docking site for chaperone-effector complexes (2), we sought to define the molecular mechanism by which SsaN captures these complexes to initiate translocation. To do so, we solved the structure of a N-terminally truncated variant of SsaN, SsaNΔ1–89, at a resolution of 2.1 Å (PDB 4NPH). The N-terminally truncated variant of SsaN was needed to generate enough soluble protein for crystallization trials, and its structure was determined by molecular replacement with the orthologous T3SS ATPase from enteropathogenic E. coli, EscN (26). A summary of the crystallographic data and refinement statistics is shown in Table 1. SsaNΔ1–89 contains two domains, a central domain (residues 90–331) and a C-terminal domain (residues 332–433). The central domain is highly similar to other ATPase catalytic domains with a mixed α/β Rossmann structure. The active site is located within the C-terminal domain.

Crystal Structure of SsaN—Crystals of SsaN were grown at 20 °C using the hanging drop vapor diffusion method. SsaN (2 μl of 1.7 mg/ml in 20 mM Tris, pH 7.5, 0.1 M potassium chloride, and 0.01 M tris(2-carboxyethyl)phosphine) was mixed with 1 μl of crystallization solution (0.5 M ammonium sulfate, 0.1 M sodium citrate tribasic dihydrate, pH 5.6, and 10% (v/v) Jeffamine M-600) and 0.2 μl of 0.1 M L-proline. The drops were initially dehydrated against 500 μl of 1.5 M ammonium sulfate. Following nucleation, the drops were transferred over successively higher concentrations of well solution to a final concentration of 4 M ammonium sulfate. Diffraction data were collected at a wavelength of 1.1 Å on the X25 beamline of the National Synchrotron Light Source at Brookhaven National Laboratory. The data were processed and scaled with HKL2000 (37) to 2.1 Å. A search model was generated from the structure of EscN from E. coli (PDB entry 2O8M) using the chainsaw algorithm (38, 39), which was used to solve the structure of SsaN by molecular replacement using PHASER from the PHENIX software package (40). Model building and refinement were carried out through multiple iterations of Coot (41) and PHENIX-Refine until R values and geometry statistics reached suitable ranges (Table 1).

Molecular models for hexameric SsaN and SrcA-SsaN were generated using the structure similarity-matching algorithm within Coot (41, 42). A hexamer of SsaN was modeled based on the F1-ATPase structure. SrcA was then modeled onto the surface of SsaN based on structural similarity between SrcA and the γ subunit of F1-ATP synthase. Protein interaction analysis for the model of SrcA-SsaN was performed using PISA (protein interfaces, surfaces, and assemblies program) (43).
A at the end of boxes for both SsaN and EscN being highly conserved (Walker composed of five helices (Fig. 2A). ATPase domains from SsaN and F$_1$-ATPases (PDB 1E79) (46) are strikingly similar and can be structurally aligned with a root mean square deviation of ~1.9 Å for Co atoms spanning 225 residues. SsaN homologues have been shown to exist as hexamers (30, 47), and SsaN can be readily modeled into a hexamer conformation based on the structure of the F$_1$-ATPase (Fig. 2B). In addition to Walker boxes for both SsaN and EscN being highly conserved (Walker A at the end of α2 and Walker B within β9), the arginine finger motif containing Arg$^{255}$, which contributes to ATP binding and possibly hexamer formation (48), is also well conserved.

Despite SsaN and EscN sharing nearly identical secondary structure and aligning with a root mean square deviation of 1.2 Å, two important differences are apparent. The first relates to alterations observed within the C-terminal domain (residues 377–393) (Fig. 3a). A point mutation (V393P) introduced in this region of EscN to facilitate structure determination (26) results in a partial disruption and unraveling of α10 compared with SsaN. This difference is particularly interesting because it corresponds to a change leading to a secretion-deficient protein and occurs in a region predicted to be important for chaperone binding (26). The possibility that the structure of SsaN represents a secretion-competent conformation is further supported by the fact that SsaN and the F$_1$-ATPase (but not EscN) adopt an identical helical structure for α10 (Fig. 3b). Importantly, this helix in the F$_1$-ATPase makes direct contact with the γ subunit of the ATP synthase complex (Fig. 3b), further implicating this region as important for mediating protein-protein interactions.

A second difference observed between SsaN (residues 268–283) and EscN results from partial unwinding of one, SrcA, for which structural information is available. During this process, it was observed that the structure of SrcA retains some structural similarity to the γ subunit of the ATP synthase. Because the γ subunit directly interacts with the analogous α10 region in the F$_1$-ATPase, a model for the SrcA-SsaN interaction was constructed based on structural similarity between SrcA and the γ subunit. Docking was performed using the secondary structure matching algorithm available within Coot (41, 42). A single monomer of SrcA aligned with the γ subunit with a root mean square deviation of 2.9 Å over 80 residues (Fig. 4A). The resulting model for hexameric SsaN interacting with a dimer of SrcA (Fig. 4B) covers a calculated (PISA) surface area of 1450 Å$^2$ involving asymmetric interactions with both subunits of SrcA and four of six subunits from SsaN. Only two steric clashes involving main chain atoms within flexible loop regions were

A competitive infections between wild-type and ΔssaN strains in C57BL/6 mice were conducted with a 1:1 mixture of both strains, and competitive index values were determined 72 h postinfection. Each filled circle represents an individual mouse (n = 5), and the geometric means are indicated by the horizontal lines. B, representative images of effector substrates that required ssaN for secretion (left panels) and those that were secreted in the absence of ssaN (right panels). Wild-type (wt) and ΔssaN strains were grown in LPM (low phosphate, low magnesium) medium, and proteins were detected in the whole cell lysate (pellet) and secreted fractions by Western blot analysis. DnaK served as a loading control and confirmed that there was no whole cell contamination in the secreted fractions. The possibility that the structure of SsaN represents a secretion-competent conformation is further supported by the fact that SsaN and the F$_1$-ATPase (but not EscN) adopt an identical helical structure for α10 (Fig. 3b). Importantly, this helix in the F$_1$-ATPase makes direct contact with the γ subunit of the ATP synthase complex (Fig. 3b), further implicating this region as important for mediating protein-protein interactions. A second difference observed between SsaN (residues 268–283) and EscN results from partial unwinding of one, SrcA, for which structural information is available. During this process, it was observed that the structure of SrcA retains some structural similarity to the γ subunit of the ATP synthase. Because the γ subunit directly interacts with the analogous α10 region in the F$_1$-ATPase, a model for the SrcA-SsaN interaction was constructed based on structural similarity between SrcA and the γ subunit. Docking was performed using the secondary structure matching algorithm available within Coot (41, 42). A single monomer of SrcA aligned with the γ subunit with a root mean square deviation of 2.9 Å over 80 residues (Fig. 4A). The resulting model for hexameric SsaN interacting with a dimer of SrcA (Fig. 4B) covers a calculated (PISA) surface area of 1450 Å$^2$ involving asymmetric interactions with both subunits of SrcA and four of six subunits from SsaN. Only two steric clashes involving main chain atoms within flexible loop regions were
present. Regions within SsaN predicted to be important for mediating this interaction include 385–397 and 401–405; however, 385–397 contributes the majority of the interaction with SrcA and involves four of six subunits within a SsaN hexamer (Fig. 4, B and C). In SrcA, the modeled regions include the following: chain A, 25–28, 42–48, and 85–88; chain B, 33–37, 64–79, and 115–125 (Fig. 4, C and D). These interaction interfaces are more distributed compared with SsaN and involve multiple, weaker contacts.

Experimental Validation of the Chaperone-T3SS ATPase Interface—The C-terminal region of T3SS ATPases has been implicated in chaperone binding (2, 26, 49); however, there has been limited work defining the precise chaperone interaction site. Based on our crystal structure of SsaN, we had identified two potential regions on SsaN that may mediate an interaction with a chaperone, residues 268–283 and 377–393. Given that our model of SrcA placed onto SsaN suggested that region 377–393 and not 268–283 would serve as the chaperone docking site, we first examined the contribution of these regions to chaperone docking in vitro. Given that T3SS ATPases can recognize both unladen chaperones (2, 44) and chaperone-effector complexes (2, 44), we focused our pull-down assays on the interaction between SsaN and unladen SrcA. As shown in Fig. 5A, a deletion within residues 268–283 (ΔV273-E276) did not disrupt binding of SsaN to SrcA. In contrast, mutation of Val379 in the 377–393 region completely abolished the interaction.
FIGURE 3. Structural differences between SsaN and EscN. a, disruption of the C-terminal α10 in EscN (blue) compared with SsaN (yellow). b, SsaN (yellow) and the F1-ATPase (light gray) adopt an identical structure in α10 compared with EscN. c, difference between α6 and α7 of SsaN (yellow) and EscN (blue). The dashed line indicates the disordered region that is absent from the structure.

(FIG. 5A). This finding implicates region 377–393 of SsaN as the binding site for SrcA and confirms the importance of the C-terminal region of T3SS ATPases in chaperone docking.

Structural comparison of EscN-V393P and SsaN suggested that a V379P mutation on SsaN would introduce a disruption toward the end of α10 and the region connecting α10 to α11, making it unsuitable for SrcA binding. Because a proline for valine substitution within this helix is expected to indirectly disrupt the chaperone interaction site, we sought to more precisely define the chaperone binding region by examining the contribution of residues at the end of α10 (Leu\(^{381}\)–Leu\(^{385}\)) and in the loop connecting α10 to α11 (Gln\(^{389}\)) to SrcA binding. Mutation of the region Leu\(^{381}\)–Leu\(^{385}\) on SsaN did not inhibit SrcA binding in the in vitro pull-down assay, whereas a Q389K mutation reduced SrcA binding to SsaN to less than half of wild-type SsaN (Fig. 5B). This result, which is in agreement with our model, implicates the loop region connecting α10 and α11 as the critical site for chaperone binding. The structural integrity of all SsaN variants was confirmed by measuring their ATPase activity as done previously for InvC variants (31). The ATPase activity of the SsaN variants was not different from wild type; however, a Walker motif mutant (K168E) was significantly impaired (Fig. 5C). We further confirmed the structural integrity of the SsaN variants that were impaired for SrcA binding by circular dichroism (Fig. 5D).

To validate the biological significance of the chaperone-ATPase interaction, we generated Salmonella strains in which the chromosomal copy of ssaN was replaced with ssaN containing either the V379P or Q389K mutation and competed these mutants against wild-type bacteria in mouse infections. The bacterial strain with the SsaN-V379P variant, which was incapable of chaperone binding in vitro but catalyzed ATP hydrolysis at wild-type levels (Fig. 5C) was as defective as an ssaN deletion mutant during host infection (Fig. 5E). The SsaN-Q389K mutant, which was only partially impaired for SrcA binding in vitro, had statistically attenuated in vivo fitness, albeit more similar to wild-type (Fig. 5E). These data establish that chaperone-T3SS ATPase interactions are a key molecular interface for bacterial fitness in the host environment.

We next examined the contribution of residues Asn\(^{26}\), Arg\(^{27}\), Trp\(^{74}\), Arg\(^{117}\), Glu\(^{220}\), and His\(^{224}\) on SrcA to the chaperone-ATPase interface because these residues were predicted to be important for SsaN binding based upon our model. The N26A/R27A and R117A/E120A/H124A mutants were some of the most impaired variants for SsaN binding, whereas individual mutation of the residues in SrcA chain B (65–79 and 115–125) had varying effects on the SrcA-SsaN interaction (Fig. 6A). Because neither chain A nor chain B mutations were sufficient to completely abolish SrcA binding, probably because SrcA forms a dimer and both regions contact SsaN, we created SrcA variants harboring combined mutations in both chains A and B (N26A/R27A with R117A, E120A, or H124A). These SrcA variants were defective for interacting with SsaN (Fig. 6B). Our model had predicted that introduction of these mutations in SrcA would have no effect on chaperone binding to effector cargo. To examine this experimentally and to verify that our combined mutants were still functional, we examined the binding of these SrcA mutants to the effector cargo PipB2. As shown in Fig. 6C, all SrcA variants retained full functionality for binding to PipB2. Taken together, these findings define the chaperone-T3SS ATPase interface and validate our structural model of the chaperone-SsaN interaction.

The universal importance of chaperone-T3SS ATPase interactions for effector secretion suggests that the C-terminal chaperone-binding domain in the ATPases may be highly conserved among Gram-negative pathogens. To investigate this, a sliding window approach was used to determine the conservation in the chaperone-binding domain of SsaN to seven orthologs in other Gram-negative bacteria. As shown in Table 3 and Fig. 7, the chaperone-binding domain of SsaN is highly conserved with respect to the corresponding region in EscN (E. coli), CdsN (Chlamydia), PscN (Pseudomonas), and YscN (Yersinia). The level of conservation in this region is similar to...
the conserved Walker boxes involved in ATP hydrolysis, whereas other regions of the proteins are less conserved (Fig. 7). Taken together with the structural work, these experiments solidified the importance of the C-terminal region of SsaN in chaperone docking.

DISCUSSION

The interaction that occurs between chaperones and T3SS ATPases is critical for ensuring that effector substrates are efficiently delivered to the secretion apparatus for translocation. Despite its importance, there has been limited work defining the precise chaperone-T3SS ATPase interaction site. In this study, we employed a combination of structural and biochemical work to elucidate the binding site of the multicargo chaperone, SrcA, on the T3SS-2 ATPase, SsaN.

We first quantified the contribution of SsaN to virulence and effector secretion. The fitness defect observed for the ssaN mutant was in agreement with the strong virulence attenuation phenotypes that have been reported for other T3SS ATPase mutants (31, 32); however, the dispensability of SsaN for the secretion of a subset of SPI-2 effectors was surprising, considering that, to date, T3SS ATPases have been shown to be essential for effector secretion (26, 31, 33). Effectors for which SsaN was dispensable for secretion were found to be targeted to the T3SS-1, a finding which is not unprecedented because PipB2 has been shown to be translocated by both the T3SS-1 and T3SS-2 (50). Most importantly, the secretion pattern of effectors highlighted a difference in the targeting and recognition of SPI-2 effectors by the T3SSs. Many of the effectors encoded outside of the SPI-2 locus do not have known cognate chaperones and are secreted by both the T3SS-1 and T3SS-2. By contrast, all of the SPI-2-encoded translocator and effector substrates have an associated chaperone and are strictly reliant on the SPI-2 T3SS system for secretion. Chaperones may therefore play a crucial role in the targeting and recognition process.

A comparison of SsaN with the E. coli T3SS ATPase EscN revealed substantial similarity between the two structures, with the most notable differences localized to the C-terminal region. The C-terminal region of T3SS ATPases had previously been implicated in chaperone binding, largely based on the demonstration that an InvC-L376P mutant is unable to bind a SicP-SptP complex (2). In the structural characterization of EscN, Zarivach et al. (26) noted that the C-terminal region of EscN was shorter than that of the F1-ATPase, which led them to
Our structural work and model of ScaA bound to SsaN raise interesting questions in regard to effector secretion. First, the high degree of sequence conservation of the chaperone binding regions on T3SS ATPases suggests that this site is under selection. Despite this conservation, SsaN and EscN adopt alternate conformations in their chaperone docking sites with the end of α10 of SsaN being replaced by a coil between α10 and α11 in EscN. It is possible that these slight structural differences represent adapted conformations tailored to the chaperone and effector repertoire within a given species. Second, the residues that define the chaperone-T3SS ATPase interaction facilitate only multiple, weak contact points between SsaN and ScaA, and binding of the chaperone to the T3SS ATPase involves asymmetric interactions. Such an interaction may allow for greater flexibility in chaperone binding and uncoupling from the T3SS ATPase before and during ATP hydrolysis, a process that must occur to energize chaperone dissociation and effector unfolding (2). Finally, assuming that the chaperone-bound T3SS ATPase is oriented to the membrane in the same manner as the $F_1$-ATPase, it seems unlikely that an unfolded effector would be passed through the center of the T3SS ATPase hexameric channel. This is because the chaperone-ATPase interaction site is proximal to the inner membrane and not oriented toward the distal end of SsaN, where the channel opening presents itself. Furthermore, in the flagellar T3SS that has many structural features in common with the virulence-associated T3SS, FliJ is similar to the γ subunit of the $F_0$-$F_1$-ATP synthase.
and was shown to occupy the central channel of the hexameric ATPase FliI (51). Therefore, it seems more probable that an unfolded effector would be passed to another component of the export apparatus to facilitate the secretion process.

T3SS ATPases have been explored as targets for novel anti-virulence drugs (52); however, targeting the active site of these enzymes may be challenging due to their conservation with eukaryotic enzymes. Peptide mimetics have proven useful in blocking protein interactions in the chlamydial T3SS, specifically for the interaction of CdsL with the ATPase, CdsN (53). Because mutation of the chaperone-binding site on SsaN resulted in severe virulence defects, an alternative target may be the essential interaction between chaperones and the T3SS ATPase. Furthermore, the high degree of conservation in the

![Diagram](image)

**FIGURE 6.** Key residues on SrcA that contribute to the chaperone-T3SS interface. A and B, interactions of wild-type FLAG-tagged SsaN with the individual SrcA chain A and B mutants (A) or combined chain mutants (B) were examined using an in vitro pull-down assay. Eluted proteins were subjected to Western blotting with the indicated antibodies. wt, wild-type SrcA. Quantifications of SsaN/SrcA ratios for input and elution fractions are shown. C, protein interaction studies between the indicated His-tagged SrcA mutants and wild-type FLAG-tagged PipB2. Proteins bound to Ni-NTA-agarose beads were eluted and subjected to Western blot analysis with anti-His and anti-FLAG antibodies. wt, wild-type SrcA. Quantifications of PipB2/SrcA ratios are shown.

**TABLE 3**

Conservation of chaperone binding domain among T3SS ATPases in Gram-negative pathogens

| Protein                  | Chaperone binding domain identity to SsaN | Overall Identity to SsaN |
|-------------------------|-------------------------------------------|--------------------------|
| EscN (E. coli)           | 84.6 (%)                                   | 49.0 (%)                 |
| CdsN (Chlamydia)         | 76.9 (%)                                   | 46.8 (%)                 |
| PscN (Pseudomonas)       | 76.9 (%)                                   | 51.9 (%)                 |
| YscN (Yersinia)          | 76.9 (%)                                   | 52.1 (%)                 |
| BscN (Bordetella)        | 69.2 (%)                                   | 48.5 (%)                 |
| InvC (Salmonella)        | 46.2 (%)                                   | 41.8 (%)                 |
| FliI (Flagellar T3SS)    | 30.8 (%)                                   | 42.7 (%)                 |

* Percentages were determined by calculating the number of identical residues between the putative chaperone binding domain of SsaN and the corresponding regions in other T3SS ATPases (window size of 13).

* Overall identity to SsaN indicates the total number of identical residues between the amino acid sequences of SsaN and other T3SS ATPases.
chaperone binding region suggests that a peptide mimetic blocking the chaperone-T3SS ATPase interaction would probably be active against a wide range of Gram-negative pathogens.

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