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Tandem Translation Generates a Chaperone for the *Salmonella* Type III Secretion System Protein SsaQ*[S]

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Background: The C-ring has a crucial role in bacterial type III secretion.
Results: *Salmonella ssaQ* produces two proteins by tandem translation: a short protein binds to its corresponding region within the larger putative C-ring protein and stabilizes it.
Conclusion: SsaQ function is optimized by a novel chaperone-like protein, produced by tandem translation from its own mRNA.
Significance: The data increase the understanding of type III secretion.

Type III secretion systems (T3SSs) of bacterial pathogens involve the assembly of a surface-localized needle complex, through which translocon proteins are secreted to form a pore in the eukaryotic cell membrane. This enables the transfer of effector proteins from the bacterial cytoplasm to the host cell. A structure known as the C-ring is thought to have a crucial role in secretion by acting as a cytoplasmic sorting platform at the base of the T3SS. Here, we studied SsaQ, an FliN-like putative C-ring protein of the *Salmonella* pathogenicity island 2 (SPI-2)-encoded T3SS. *ssaQ* produces two proteins by tandem translation: a long form ($SsaQ_L$) composed of 322 amino acids and a shorter protein ($SsaQ_S$) comprising the C-terminal 106 residues of $SsaQ_L$. $SsaQ_L$ is essential for SPI-2 T3SS function. Loss of $SsaQ_S$ impairs the function of the T3SS both *ex vivo* and *in vivo*. $SsaQ_S$ binds to its corresponding region within $SsaQ_L$ and stabilizes the larger protein. Therefore, $SsaQ_L$ function is optimized by a novel chaperone-like protein, produced by tandem translation from its own mRNA species.

Following uptake or invasion of mammalian host cells, *Salmonella enterica* serovar Typhimurium (sv. Typhimurium) replicates within membrane-bound compartments known as *Salmonella*-containing vacuoles. Acidification of lumen of the *Salmonella*-containing vacuole stimulates expression of genes involved in the assembly of a multiprotein structure that spans the bacterial cell envelope, called the *Salmonella* pathogenicity island 2 (SPI-2)[2] type III secretion system (T3SS) (1–3). This T3SS secretes proteins that assemble a needle structure and a translocon pore in the vacuolar membrane (1). Sensing of the near-neutral pH of the host cell cytosol by unknown component(s) of the T3SS triggers the dissolution and loss in the bacterial cytoplasm of a T3SS-associated regulatory complex comprising three proteins: SsaL, SpiC, and SsaM (4). This relieves repression of secretion of ~25 effector proteins that are translocated across the vacuolar membrane (4). The effectors have many different functions, affecting immune signaling (5), bacterial and host cell motility (6, 7), and intracellular replication of bacteria in a variety of host cell types, including epithelial cells and macrophages (8). As a result, SPI-2 T3SS null mutants are strongly attenuated in virulence in various hosts (9–12).

ssaQ is the last gene in the *ssaMVNOPQ* operon within SPI-2 (13, 14). The predicted product of *ssaQ* is a member of the FliN/YscQ/Spa33/HrcQ family of flagellum and T3SS proteins (15). These proteins belong to the conserved essential core of these structures and are thought to constitute a cytoplasmic platform (C-ring) connected to the base of the secretion system (16, 17). In this work, we studied the *ssaQ* gene of sv. Typhimurium. We found that it produces two proteins from one transcript: a protein of the expected size and a second translational product corresponding to the C-terminal 106 amino acids. The tandem translated small protein acts as a chaperone, binding to and stabilizing the larger protein, and is important for the overall efficiency of the secretion system.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The sv. Typhimurium strains used in this study are derivatives of wild-type strain 12023 and are listed in Table 1. Bacteria were grown in LB medium supplemented with carbenicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (15 μg/ml), or tetracycline (25 μg/ml) for strains resistant to these antibiotics (Amp^r, Kan^r, Cam^r, and Tet^r, respectively). To induce SPI-2 gene expression and SPI-2-dependent secretion, bacteria were grown in magnesium minimal medium (MgM)/MES (3) at pH 5.0 with the corresponding antibiotics when appropriate.

The λ Red recombination system (18) was used to construct *ssaQ* deletion mutants HH225 and HH227 using primers ssaQd1 and ssaQd2 or primers ssaQd3 and ssaQd4, respectively. Primers are listed in the supplemental table. Chromosomal allelic exchange was used to construct $ssaQ_S$ point

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[2] The abbreviations used are: SPI-2, *Salmonella* pathogenicity island 2; T3SS, type III secretion system; MgM, magnesium minimal medium; CI, competitive index.

TABLE 1
sv. Typhimurium strains constructed and used in this study

Name	Description	Source or Ref.
12023	Wild-type	NCTC
P3F4	*ssrA*::*mTn5* in 12023 (Kan^r)	Ref. 9
HH216	*sseJ-2HA*::*cat* in 12023 (Cam^r)	Ref. 21
HH225	Δ*ssaQ*$_{18–311}$::*Kan* in 12023 (Kan^r)	This study
HH226	*sseJ-2HA*::*cat* in Δ*ssaQ*$_{18–311}$::*Kan* mutant	This study
HH227	Δ*ssaQ*$_{104–223}$::*cat* in 12023 (Cam^r)	This study
HH228	*ssaQ* replaced with *ssaQ*$_{M217L}$ in 12023	This study
HH229	*ssaQ* replaced with *ssaQ*$_{Y206oc}$ in 12023	This study
HH230	*sseJ-2HA*::*cat* in HH229	This study
HH231	*sseJ-2HA*::*cat* in HH228	This study
HH232	*ssaQ* replaced with *ssaQ*$_{M217L}$-*HA*::*Kan* in 12023	This study

mutant HH228 or *ssaQ*$_L$ point mutant HH229. Briefly, *ssaQ* was ligated into pCR2.1-TOPO (Invitrogen) using primers ssaQf and ssaQb to construct pCR-*ssaQ*, followed by site-directed mutagenesis to create pCR-*ssaQ*$_{M217L}$ and pCR-*ssaQ*$_{Y206oc}$ using primers ssaQ$_{M217L}$f and ssaQ$_{M217L}$b or primers ssaQ$_{Y206oc}$f and ssaQ$_{Y206oc}$b, respectively. Mutant versions of *ssaQ* were subcloned into suicide vector pCVD442 for transferring mutations to HH227 by conjugation, and exconjugants were selected as described previously (19). Primers ssaQHAf and ssaQdb were used to fuse DNA segments encoding the HA epitope to the 3′ termini of the coding region of *ssaQ* or *ssaQ*$_L$ in WT or HH228 strains as described previously (20).

To express *sseJ-2HA* from chromosomal DNA, *sseJ-2HA*::*cat* from HH216 (21) was transduced into different strains of sv. Typhimurium by phage P22 as described previously (22). When necessary, the *FRT*-flanked antibiotic resistance cassette was removed after transformation with pCP20 as described (18).

Plasmids—The *spiC* promoter was ligated into pWSK29 (23) using primers spiCn1 and spiCn2 to create p*spiCpr*. Plasmids p*ssaQ* and p*ssaQ*$_L$ were constructed by subcloning *ssaQ* and *ssaQ*$_{M217L}$ fragments from pCR-*ssaQ* and pCR-*ssaQ*$_{M217L}$ into p*spiCpr*. The *ssaQ*$_S$ gene was amplified using primers ssaQ$_S$f and ssaQb and ligated into p*spiCpr* to construct p*ssaQ*$_S$. The HA-tagged *ssaQ*, *ssaQ*$_{M217L}$, and *ssaQ*$_{Y206oc}$ DNA fragments were amplified from pCR-*ssaQ*, pCR-*ssaQ*$_{M217L}$, and pCR-*ssaQ*$_{Y206oc}$ using primers ssaQf and ssaQHAb and ligated into p*spiCpr* to create plasmids p*ssaQ-HA*, p*ssaQ*$_{M217L}$*-HA*, and p*ssaQ*$_{Y206oc}$*-HA*. The *ssaQ*$_{LN216}$*-HA* fragment was amplified using primers ssaQf and ssaQ$_{LN216}$HAb and ligated into p*spiCpr* to construct p*ssaQ*$_{LN216}$*-HA*. The *ssaQ*$_L$*-T7* fragment was amplified from p*ssaQ*$_{M217L}$*-HA* using primers ssaQf-SacI and ssaQT7b-SacI and ligated into p*spiCpr*, p*ssaQ*$_{M217L}$*-HA*, and p*ssaQ*$_{LN216}$*-HA* to create plasmids p*ssaQ*$_L$*-T7*, p*ssaQ*$_L$*-HA/ssaQ*$_L$*-T7*, and p*ssaQ*$_{LN216}$*-HA/ssaQ*$_L$*-T7*, respectively. Plasmid p*ssaQ*$_S$*-T7* was constructed by replacing the *cat* gene of pACYC184 (24) with *ssaQ*$_S$*-T7* following PCR amplification with primers ssaQ$_S$f and ssaQT7b. The expression of *ssaQ*$_S$*-T7* is under the control of the constitutive promoter of *cat*.

The PCR products including the promoter region of *ssaM* to the start codon of *ssaQ*$_S$ (using primers 102ssaM-KpnI and ssaQ$_{L217}$r-XbaI) or beginning from the start codon of *ssaM* to the start codon of *ssaQ*$_S$ (using primers ssaM-KpnI and ssaQ$_{L217}$r-XbaI), amplified from genomic DNA of sv. Typhimurium strain 12023, were ligated into the KpnI and XbaI sites of pFPV25, a vector carrying promoterless *gfpmut3A* (25), to create p*102ssaMQ*$_{LN217}$ and p*ssaMQ*$_{LN217}$, respectively. To express *ssaQ*$_S$ with a His$_6$ tag, the cleaved *ssaQ*$_S$ PCR product (using primers ssaQ$_S$-EcoRIf and ssaQ$_S$-XhoIb) was ligated into pET-22b (Novagen) to construct pET-*ssaQ*$_S$. Plasmids constructed as part of this study were verified by DNA sequencing and are listed in Table 2. Plasmid p*steC-2HA* was described previously (26).

Antibodies—The following primary antibodies were used for immunoprecipitation, immunofluorescence staining, and immunoblot analysis: rat anti-HA antibody (3F10, Roche Applied Science); mouse anti-T7 antibody (Novagen); mouse anti-HA monoclonal antibody HA.11 (MMS-101P, Covance); mouse anti-DnaK antibody (Assay Designs); goat anti-*Salmonella* polyclonal antibody CSA-1 (Kirkegaard & Perry Laboratories); and rabbit anti-SseB, anti-SseC, and anti-SseD polyclonal antibodies (21). Texas Red sulfonyl chloride-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories) was used for immunofluorescence microscopy. Alexa Fluor 488-conjugated donkey anti-goat antibody and Alexa Fluor 647-conjugated donkey anti-mouse antibody (Invitrogen) were used for flow cytometric analysis. The following HRP-conjugated secondary antibodies were used for immunoblot analysis: donkey anti-rabbit and sheep anti-mouse antibodies (Amersham Biosciences) and rabbit anti-rat antibody (Dako).

Preparation of Protein Fractions from Bacteria Grown in Vitro—Bacterial strains were grown in MgM/MES for secretion assays as described previously (4, 21). To ensure that protein from equal numbers of cells was analyzed, in all experiments, protein samples were adjusted to A_{600} values such that a volume corresponding to 10 ml of a culture with $A_{600} = 0.6$ was taken up in 100 μl of protein denaturing buffer for secreted and bacterial surface-associated fractions and in 600 μl of protein denaturing buffer for the total bacterial fraction.

Immunoprecipitation Assays—Volumes corresponding to 20 ml of a bacterial culture with $A_{600} = 0.6$ after 5 h of growth of sv. Typhimurium in MgM/MES at pH 5.0 were used to perform immunoprecipitation assays. Bacteria were collected by centrifugation and resuspended in a solution comprising 1 ml of 50 mM glucose, 10 mM EDTA, 4 mg/ml lysozyme, and 25 mM Tris-Cl (pH 8.0) and incubated for 5 min at room temperature to generate spheroplasts. The spheroplasts were resuspended into 1.5 ml of lysis buffer (50 mM Tris-Cl (pH 8.0, 100 mM NaCl, 1% Triton X-100, and 1 mM PMSF) and incubated on ice for 30 min with occasional mixing. The lysate was centrifuged for 10 min at 10,000 × *g*, and the supernatant was transferred into a fresh tube and incubated with 50 μl of protein G-immobilized agarose (Pierce) for 1 h to preclear the lysate. The precleared supernatant was incubated with 10 μl of rat anti-HA antibody or mouse anti-T7 antibody for 2 h at 4 °C to form antibody-antigen complexes, and 50 μl of protein G-immobilized agarose was added to the reaction and incubated for 2 h at 4 °C. The beads were collected by centrifugation at 500 × *g* for 4 min and washed four times with 700 μl of lysis buffer prior to boiling in 50 μl of SDS-PAGE sample buffer.

N-terminal Sequencing of SsaQ$_S$—The bacterial lysate of *ssaQ* p*ssaQ-HA* culture grown in MgM/MES was subjected to immunoprecipitation with rat anti-HA antibody. SsaQ$_S$-HA was recovered from the PVDF membrane and sequenced by

TABLE 2
Plasmids used in this study

Name	Description	Source or Ref.
pWSK29	pSC101 *ori*, low copy number vector, Amp^r	Ref. 23
pFPV25	Carrying promoterless *gfpmut3A*, Amp^r	Ref. 25
pCVD442	Suicide vector for DNA allelic exchange, Amp^r	Ref. 19
pACYC184	p15A *ori*, medium copy number vector, Tet^r, Cam^r	Ref. 24
p*spiCpr*	*spiC* promoter on pWSK29, Amp^r	This study
p*ssaQ*	*ssaQ* gene on p*spiCpr*, Amp^r	This study
p*ssaQ-HA*	*ssaQ-HA* gene on p*spiCpr*, Amp^r	This study
p$ssaQ_{M217L}$-*HA*	$ssaQ_{M217L}$-*HA* gene on p*spiCpr*, Amp^r	This study
p$ssaQ_{Y2060c}$-*HA*	$ssaQ_{Y2060c}$-*HA* gene on p*spiCpr*, Amp^r	This study
p*102ssaMQ*$_{LN217}$	Sequence composed of promoter region of *ssaM* to start codon of $SsaQ_S$ on pFPV25, Amp^r	This study
p$ssaMQ_{LN217}$	Sequence composed of start codon of *ssaM* to start codon of $SsaQ_S$ on pFPV25, Amp^r	This study
p$ssaQ_L$	$ssaQ_{M217L}$ gene on p*spiCpr*, Amp^r	This study
p$ssaQ_S$	$ssaQ_S$ gene on p*spiCpr*, Amp^r	This study
p$ssaQ_S$-*T7*	$ssaQ_S$-*T7* gene on pACYC184, Tet^r	This study
p$ssaQ_{LN216}$-*HA*	$ssaQ_{LN216}$-*HA* gene on p*spiCpr*, Amp^r	This study
pET-$ssaQ_S$	$ssaQ_S$ gene on pET-22b, Amp^r	This study
p$ssaQ_L$-*T7*	$ssaQ_L$-*T7* gene on p*spiCpr*, Amp^r	This study
p$ssaQ_L$-*HA*/$ssaQ_L$-*T7*	$ssaQ_L$-*T7* gene on p$ssaQ_{M217L}$-*HA*, Amp^r	This study
p$ssaQ_{LN216}$-*HA*/$ssaQ_L$-*T7*	$ssaQ_L$-*T7* gene on p$ssaQ_{LN216}$-*HA*, Amp^r	This study
p*steC-2HA*	*steC-2HA* gene with *steC* promoter on pWSK29, Amp^r	Ref. 26

Edman degradation (Protein & Nucleic Acid Chemistry Facility, University of Cambridge). The first six amino acids obtained for $SsaQ_S$ were MKFDEL.

Cross-linking and Protein Stability Assays—Escherichia coli BL21(DE3) cells containing plasmid pET-$ssaQ_S$ were cultured in LB medium and induced for 2 h at 30 °C with 1 mM isopropyl β-D-thiogalactopyranoside to express $SsaQ_S$-His_6. The bacteria were treated with DMSO (Sigma) or the cross-linker disuccinimidyl suberate (1 mM; Pierce) as recommended by the manufacturer and subjected to Western blotting with HRP-conjugated anti-His antibody (ab1187, Abcam).

For the $SsaQ_L$-HA stability assay, bacterial strains were cultured in MgM/MES at pH 5.0 for 4 h, and tetracycline was then added to a final concentration 20 μg/ml to stop protein synthesis. Samples were removed at different time points for immunoblotting.

PAGE and Immunoblot Analysis of Proteins—Protein fractions were dissolved in the appropriate volume of protein denaturing buffer and held at 100 °C for 10 min. Proteins were immediately separated on 12% SDS-polyacrylamide gels. For immunoblot analysis, proteins were transferred to Immobilon-P (PVDF) membranes (Millipore) and examined using the ECL detection system (Amersham Biosciences) under conditions recommended by the manufacturer. Incubation of membranes with primary antibodies was followed by incubation with HRP-conjugated secondary antibodies.

Cell Culture, Immunofluorescence, and Flow Cytometric Analysis—HeLa epithelial cells (clone HtTA1) were grown in DMEM (Invitrogen) supplemented with 10% FCS and 2 mM glutamine at 37 °C in 5% CO_2 and infected with exponential phase sv. Typhimurium as described previously (27).

To visualize GFP expression, HeLa cells were fixed with 3% paraformaldehyde at 5 h post-invasion. Bacteria were labeled first with antibody CSA-1 and then with Texas Red sulfonyl chloride-conjugated donkey anti-goat antibody. Images were taken on a Zeiss LSM 510 confocal laser scanning microscope.

To detect translocation of SseJ-2HA, HeLa cells infected with *Salmonella* strains for 13 h were trypsinized and then fixed with 3% paraformaldehyde. Antibodies CSA-1 and HA.11 were used to label bacteria and translocated SseJ-2HA in the presence of 0.1% saponin (Sigma), followed by labeling with secondary antibodies conjugated to Alexa Fluor 488 or 647. Approximately 1×10^5 infected cells were analyzed on a FACSCalibur cytometer (BD Biosciences), and bacteria and SseJ-2HA were detected at 525 nm in the FL1 channel and at 633 nm in the FL4 channel. Data were analyzed with FlowJo 8.8.6 software.

Bioinformatic Analysis and Homology Modeling—Homology models of $SsaQ_S$ were calculated using the Phyre protein structure prediction server (28). The homology model of $SsaQ_S$ was calculated from the primary amino acid sequence of the flagellar rotor protein FliN from *Thermotoga maritima* (29). The $SsaQ_S$ sequence was aligned, submitted to multiple secondary structure prediction, and compared against the Phyre fold library. The top 10 alignments were used to produce an ensemble of three-dimensional models. The top-scoring Phyre homology model was used as the representative monomer structure, and a final model for the dimer was assembled by superposition on the dimer of FliN. ClustalW2 was used to align the two protein sequences.

Mouse Mixed Infections—Female BALB/c mice (20–25 g) were used for competitive index (CI) studies. Four mice were inoculated intraperitoneally with a mixture of two strains comprising 500 colony-forming units of each strain in physiological saline, and the CIs were determined from spleen homogenates 96 h post-inoculation as described previously (30). The *p* value was determined by two-tailed *t* test.

RESULTS

Products of SsaQ—SsaQ is encoded by SPI-2 and shares weak overall similarity (14.6% identity and 38.5% similarity) with Spa33, the C-ring protein of the *Shigella* T3SS (16). The C-ring proteins show greater conservation in the C-terminal regions, and an alignment of this region revealed several conserved residues that are also found in SsaQ (15). To investigate the function of SsaQ in SPI-2 type III secretion, an *ssaQ* deletion mutant with the chromosomally encoded epitope-tagged effector SseJ-2HA was constructed and analyzed. In minimal medium at pH 5.0, the SPI-2 T3SS is activated and secretes high levels of translocon proteins and very low levels of effectors (3, 4). Under these conditions, secretion of the translocon protein SseB and

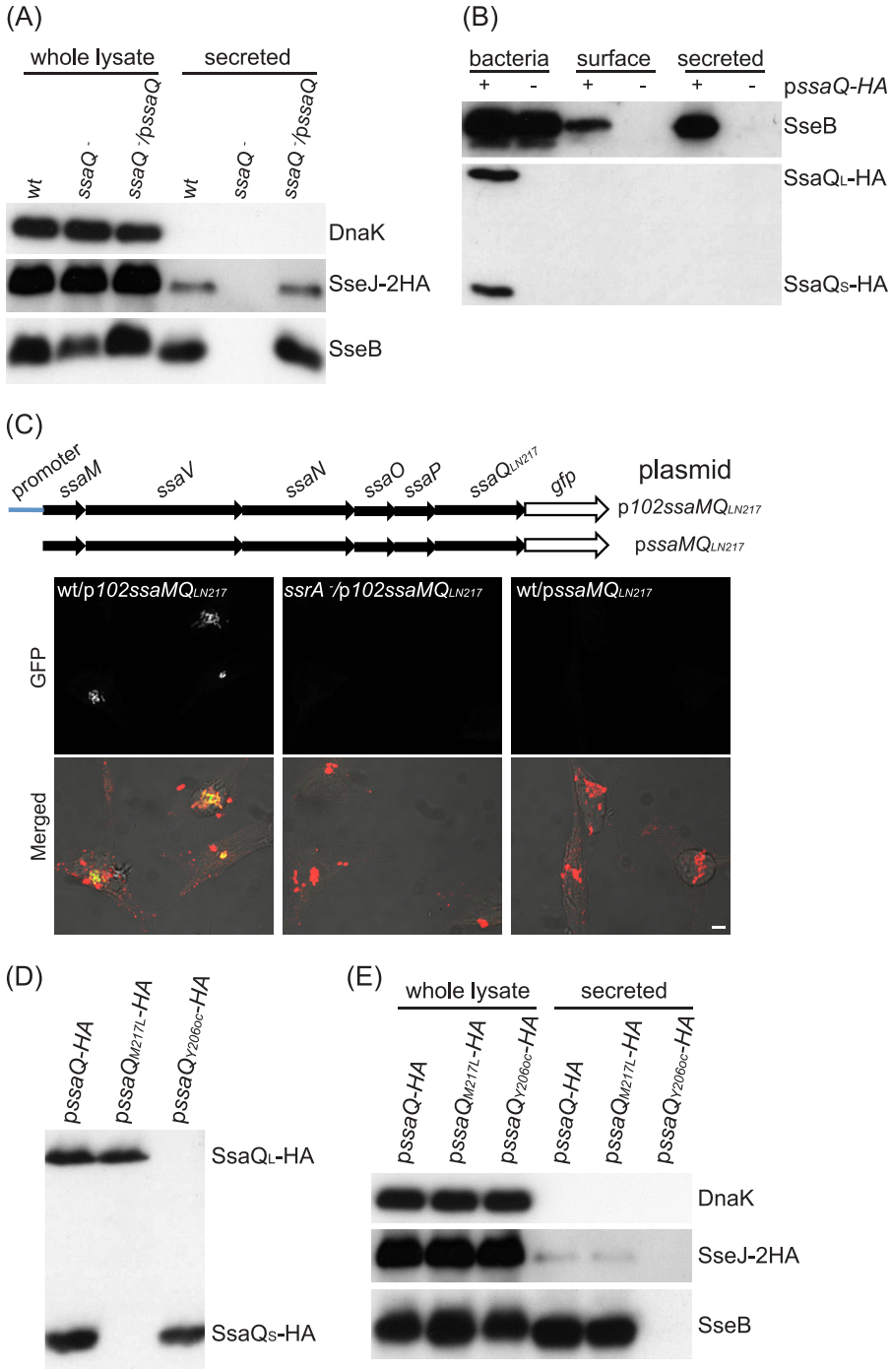

FIGURE 1. **Analysis of *ssaQ* gene products and their function.** *A*, essential role of SsaQ in secretion of SseB and SseJ. Bacterial strains expressing SseJ-2HA from the chromosome were grown in MgM/MES at pH 5.0 for 5 h, and whole bacterial cell lysates and secreted fractions were subjected to immunoblot analysis. The intrabacterial protein DnaK was used as a control. *B*, neither $SsaQ_L$-HA nor $SsaQ_S$-HA was secreted or presented on the bacterial surface. Plasmid p*ssaQ-HA* was transformed into the *ssaQ* mutant (HH225) for analysis. Bacterial surface proteins were extracted with *n*-hexadecane. *C*, the promoter upstream of *ssaM* is the only one driving expression of the *ssaMVNOPQ* operon. The schematic illustrates the plasmids used in the transcriptional assay. The confocal micrographs show HeLa cells infected with the indicated bacterial strains for 5 h. *Green*, GFP; *red*, CSA-1-labeled bacteria. *Scale bar* = 5 μm. *D*, $SsaQ_S$-HA is a translated product rather than a cleavage product of $SsaQ_L$-HA. The *ssaQ* mutant (HH225) was transformed with different plasmids and grown in MgM/MES at pH 5.0 for 5 h, and whole bacterial cell lysates were subjected to immunoblot analysis. *E*, $SsaQ_L$, but not $SsaQ_S$, is essential for secretion of SseB and SseJ. The *ssaQ* mutant expressing SseJ-2HA from chromosomal DNA (HH226) was transformed with the indicated plasmids and used for analysis.

SseJ-2HA from the mutant strain was undetectable, and this phenotype was complemented by introduction of a plasmid expressing wild-type SsaQ (Fig. 1*A*).

As well as being a component of the C-ring, Spa33 has been proposed to be a secreted substrate of the T3SS (31). To determine whether SsaQ is secreted, a plasmid expressing C-terminally HA-tagged SsaQ (SsaQ-HA) was introduced into the *ssaQ* deletion mutant. SsaQ-HA was functional, as shown by its ability to restore SseB secretion in minimal medium at pH 5.0 (Fig. 1*B*). However, under these conditions, SsaQ-HA was not detected in culture supernatants or on the bacterial cell surface (Fig. 1*B*). Interestingly, two proteins were detected in the bac-

terial lysate with an anti-HA antibody. One corresponds to the expected mass of SsaQ (36 kDa), and the other is ~12 kDa. The small protein was not due to the overexpression of SsaQ-HA from the plasmid, as it was also detected when SsaQ-HA was expressed from chromosomal DNA (data not shown). To establish the identity of the small protein, it was gel-purified and subjected to N-terminal sequencing. This revealed that it comprises the C-terminal 106 amino acids of SsaQ, with Met-217 of SsaQ at its N terminus. The full-length protein was designated $SsaQ_L$, and the smaller protein was designated $SsaQ_S$.

The expression of *ssaQ* is controlled by a promoter of the 5.4-kb *ssaMVNOPQ* operon (13, 14). To determine whether $SsaQ_S$ is derived from an independent transcript, DNA fragments either including the 102-bp promoter sequence upstream of *ssaM* or beginning from the start codon of *ssaM* to the putative start codon of $ssaQ_S$ were ligated into a *gfp* reporter plasmid to generate plasmids p*102ssaMQ*$_{LN217}$ and p*ssaMQ*$_{LN217}$ respectively (Fig. 1*C*). The plasmids were transformed into sv. Typhimurium strains, which were then used to infect HeLa cells for 5 h. There was no detectable GFP fluorescence in bacterial cells carrying the plasmid lacking the 102-bp promoter sequence. GFP production was observed in >90% of bacterial cells containing the 102-bp promoter sequence and was completely dependent on the SPI-2 two-component regulatory system SsrA-B (Fig. 1*C*) (32). These results indicate that both $SsaQ_L$ and $SsaQ_S$ are translated from the *ssaMVNOPQ* transcript.

To determine whether $SsaQ_S$ is a cleavage product of $SsaQ_L$ or is translated separately, *ssaQ-HA* was subjected to site-directed mutagenesis, and the effects were examined following production of mutant proteins from a plasmid in an sv. Typhimurium *ssaQ* null mutant strain grown in minimal medium at pH 5.0. If $SsaQ_S$ is the product of cleavage of $SsaQ_L$, then it should not be produced if $SsaQ_L$ is truncated before Met-217. Therefore, the codon for Tyr-206 of $SsaQ_L$-HA was replaced with a stop codon (ochre mutation, TAA). As expected, $SsaQ_L$-HA was not detected upon expression of $SsaQ_{Y206oc}$-HA, but $SsaQ_S$-HA was still produced by this mutant (Fig. 1*D*). Substituting the codon for Met-217 (the putative start codon for $SsaQ_S$) with a leucine codon ($SsaQ_{M217L}$) resulted in an absence of $SsaQ_S$-HA but not $SsaQ_L$-HA (Fig. 1*D*). These results show that $SsaQ_S$ is not a product of cleavage of $SsaQ_L$ and that $SsaQ_L$ and $SsaQ_S$ are translated independently from the same mRNA species. Consistent with this, there is a purine-rich sequence upstream of the start codon of $ssaQ_S$ (AGAGGATAACACGATG), which is likely to be the Shine-Dalgarno sequence for translational initiation (33).

We next used these strains to examine the involvement of $SsaQ_L$ and $SsaQ_S$ in SPI-2 T3SS function. In the absence of $SsaQ_L$ (the *ssaQ* null mutant strain expressing $SsaQ_{Y206oc}$-HA from a plasmid), there was no detectable secretion in minimal medium at pH 5.0 of either the translocon protein SseB or the effector SseJ (Fig. 1*E*). However, the absence of $SsaQ_S$ (the *ssaQ* null mutant strain expressing $SsaQ_{M217L}$-HA from a plasmid) did not noticeably affect secretion of SseB or SseJ at pH 5.0 (Fig. 1*E*). This result indicates that $SsaQ_{M217L}$-HA is functional and that $SsaQ_L$ is essential for the SPI-2 T3SS, whereas $SsaQ_S$ is not.

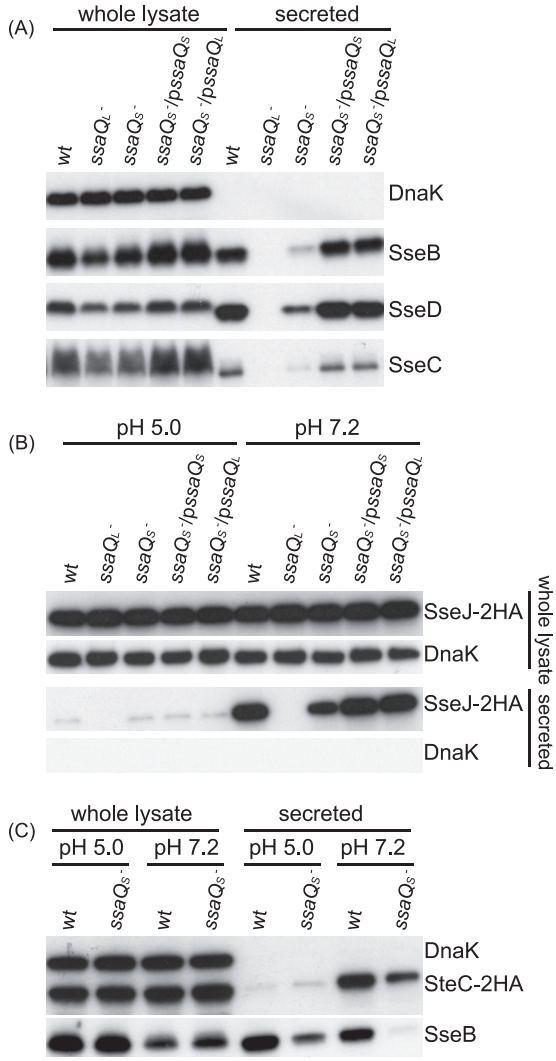

FIGURE 2. **Role of $SsaQ_S$ in secretion of translocon and effector proteins.** The *ssaQ* gene of the wild-type strain of sv. Typhimurium was replaced with $ssaQ_{Y206oc}$ or $ssaQ_{M217L}$ to create the $ssaQ_L$ (HH229) or $ssaQ_S$ (HH228) mutant, respectively, and used for secretion assays. *A*, secretion of translocon proteins. Bacterial strains were grown for 5 h in MgM/MES at pH 5.0 for analysis. *B*, secretion of effector SseJ-2HA upon pH shift. Strains expressing SseJ-2HA from chromosomal DNA were grown for 4 h in MgM/MES at pH 5.0 to activate SPI-2 T3SS and then changed to MgM/MES at the indicated pH and incubated for another 1.5 h. *C*, secretion of effector SteC-2HA upon pH shift. Strains carrying plasmid p*steC-2HA* were used for pH shift analysis. Secretion of translocon protein SseB was used as an additional control.

$SsaQ_S$ Is Required for Efficient Secretion of Translocon and Effector Proteins—To further investigate the possible function of $SsaQ_S$, the point mutation for M217L was introduced into the chromosomal *ssaQ* gene to create a mutant in which $SsaQ_L$ is produced as a result of expression from chromosomal DNA but in which $SsaQ_S$ is lacking. In minimal medium at pH 5.0, the mutant displayed highly reduced secretion of all three translocon proteins: SseB, SseC, and SseD (Fig. 2*A*). The decreased secretion of these proteins in the $ssaQ_S$ mutant was restored to wild-type levels by introduction of a plasmid expressing either $ssaQ_S$ or $ssaQ_L$ (Fig. 2*A*). The ability of overexpressed $SsaQ_L$ to compensate for loss of $SsaQ_S$ was also evident when $ssaQ_L$ was overexpressed in the *ssaQ* mutant (Fig. 1*E*). These results indicate that $SsaQ_S$ is required for the efficient secretion of translo-

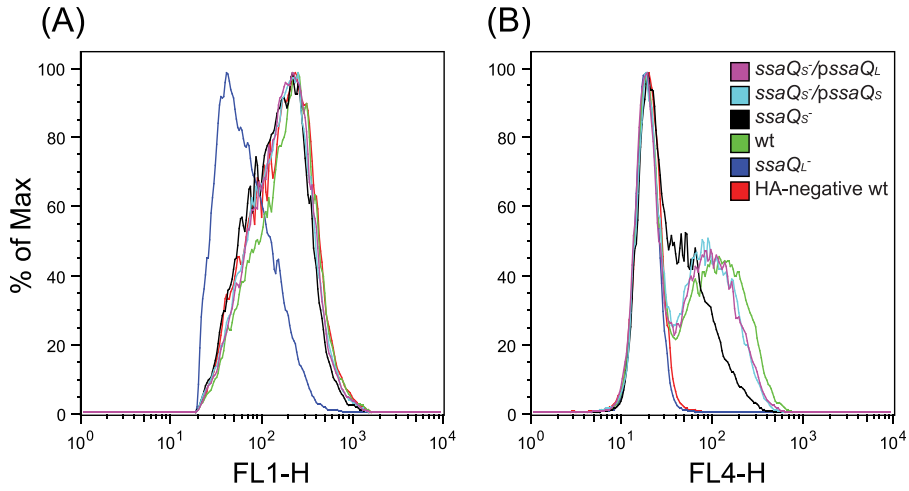

FIGURE 3. **Flow cytometric analysis of translocation of SseJ-2HA.** HeLa cells were infected for 13 h with bacterial strains expressing SseJ-2HA from chromosomal DNA except for the HA-negative wild-type strain and labeled with antibodies for flow cytometric assay. *A*, intracellular bacteria were detected with anti-*Salmonella* antibody (FL1). *B*, translocated SseJ-2HA was detected with anti-HA antibody (FL4).

con proteins and that overexpression of $SsaQ_L$ can compensate for the loss of $SsaQ_S$.

To examine the potential role of $SsaQ_S$ in secretion of effector proteins, the secretion of epitope-tagged SseJ-2HA was investigated following shift of ambient pH from 5.0 to 7.2, which stimulates effector secretion from the SPI-2 T3SS (4). Bacterial strains were first grown in minimal medium at pH 5.0 for 4 h to activate the T3SS and then incubated in the same medium at pH 5.0 or 7.2 for 1.5 h. Secreted fractions and whole cell lysates were subjected to SDS-PAGE and immunoblotting. In response to the pH shift, the wild-type and *ssaQ*$_S$ mutant strains complemented with either *ssaQ*$_L$ or *ssaQ*$_S$ displayed greatly enhanced and similar levels of SseJ-2HA secretion, respectively (Fig. 2*B*). In contrast, quantification of the amount of SseJ-2HA secreted by the *ssaQ*$_S$ mutant showed that it was ~44% of the wild-type level (Fig. 2*B*). Mutation of *ssaQ*$_S$ caused a similar reduction in the secreted levels of another effector, SteC-2HA (Fig. 2*C*). These findings indicate that the translocon-to-effector switch mediated by the SsaL-SpiC-SsaM complex in response to pH shift (4) still occurs in the absence of $SsaQ_S$, but the overall efficiency of secretion through the SPI-2 T3SS is reduced *in vitro*.

SsaQ$_S$ *Is Required for Efficient Translocation of Effectors*—We next examined whether $SsaQ_S$ contributes to effector translocation in infected cells. To do this, HeLa cells were infected for 13 h with different strains expressing the epitope-tagged effector SseJ-2HA from the bacterial chromosome, and the levels of translocation were quantified by flow cytometry. The *ssaQ*$_L$ mutant was unable to translocate SseJ-2HA. The levels of translocated SseJ-2HA from the *ssaQ*$_S$ mutant bearing the complementing plasmid p*ssaQ*$_S$ or p*ssaQ*$_L$ were similar to those from the wild-type strain, but the *ssaQ*$_S$ mutant translocated noticeably less SseJ-2HA (Fig. 3). Quantification of the amount of SseJ-2HA translocated by the *ssaQ*$_S$ mutant showed that it was ~50% of the wild-type level. We concluded that $SsaQ_S$ is required for efficient translocation of SPI-2 T3SS effectors in infected host cells.

SsaQ$_S$ *Interacts with and Stabilizes SsaQ*$_L$—The observation that overexpression of $SsaQ_L$ compensates for the loss of $SsaQ_S$ suggested that $SsaQ_S$ might function as a chaperone, stabilizing $SsaQ_L$. To test if $SsaQ_S$ interacts with $SsaQ_L$, the *ssaQ* mutant strain containing plasmid p*ssaQ-HA* (which produces both $SsaQ_L$-HA and $SsaQ_S$-HA) and either p*ssaQ*$_S$*-T7* or the empty vector (pACYC184) was grown in minimal medium at pH 5.0. Lysates were incubated with an antibody against T7 to immunoprecipitate $SsaQ_S$-T7. $SsaQ_L$-HA and $SsaQ_S$-HA were not co-immunoprecipitated in the negative control strain containing plasmids p*ssaQ-HA* and pACYC184. However, both $SsaQ_L$-HA and $SsaQ_S$-HA were co-immunoprecipitated from the strain containing p*ssaQ-HA* and p*ssaQ*$_S$*-T7* (Fig. 4*A*). This result suggested that $SsaQ_S$ interacts with the C-terminal domain ($SsaQ_S$ region) of $SsaQ_L$. To test this, a plasmid expressing the N-terminal 216 amino acids of $SsaQ_L$ (*pssaQ*$_{LN216}$*-HA*) was transformed into the *ssaQ* mutant containing plasmid p*ssaQ*$_S$*-T7*, and following growth in minimal medium at pH 5.0, bacterial lysates were subjected to immunoprecipitation with an antibody against the HA tag. As a positive control, this antibody was shown to co-immunoprecipitate $SsaQ_S$-T7 in the strain containing plasmids p*ssaQ-HA* and p*ssaQ*$_S$*-T7* (Fig. 4*B*). However, the antibody did not immunoprecipitate $SsaQ_S$-T7 in the *ssaQ* mutant containing *pssaQ*$_{LN216}$*-HA* and p*ssaQ*$_S$*-T7* (Fig. 4*B*). In addition, a cross-linking experiment showed that $SsaQ_S$ homodimerizes following its production in *E. coli* (Fig. 4*C*). These experiments provided evidence that $SsaQ_S$ interacts with the corresponding region of $SsaQ_L$. Next, we tested if $SsaQ_L$ oligomerizes in the absence of $SsaQ_S$. To do this, plasmids expressing $SsaQ_L$-T7 and either $SsaQ_L$-HA or $SsaQ_{LN216}$-HA were transformed into the *ssaQ* mutant and subjected to immunoprecipitation with the anti-HA antibody. $SsaQ_L$-T7 was co-precipitated in the presence of $SsaQ_L$-HA but not by the N-terminal 216 amino acids of $SsaQ_L$ (Fig. 4*D*). Therefore, the $SsaQ_S$ region of $SsaQ_L$, but not $SsaQ_S$ itself, is required for $SsaQ_L$ to oligomerize.

To assess the stability of $SsaQ_L$ in the absence of $SsaQ_S$, a bacterial strain was constructed in which the chromosomal *ssaQ* gene was modified to express $SsaQ_{M217L}$-HA but not $SsaQ_S$. This strain was then transformed with p*ssaQ*$_S$ or the empty vector (pWSK29). The transformants were grown in

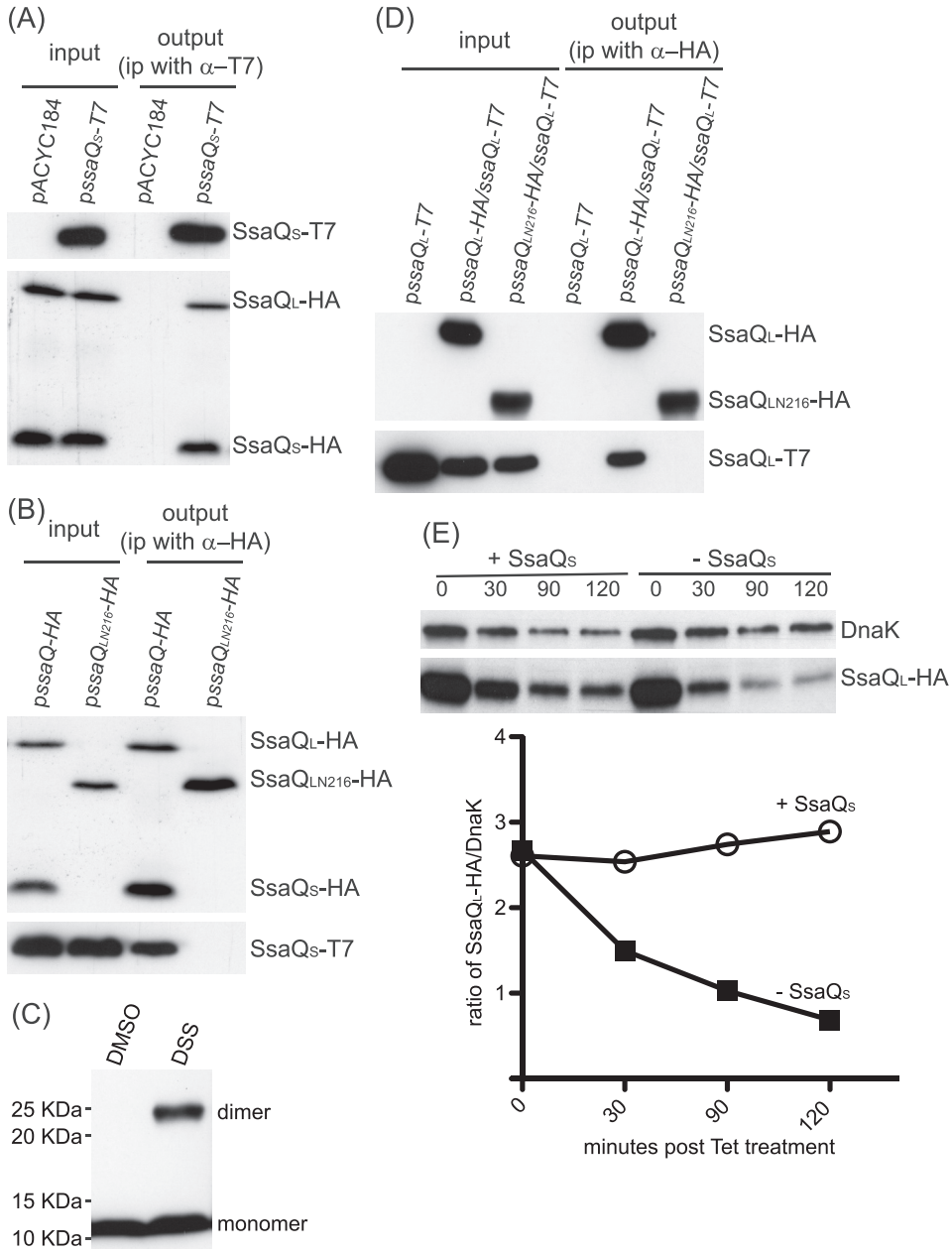

FIGURE 4. **$SsaQ_S$ functions as a chaperone for $SsaQ_L$.** *A*, $SsaQ_S$ interacts with $SsaQ_L$ and itself. Bacterial strain *ssaQ pssaQ-HA* was cotransformed with p*ssa*Q_S*-T7* or vector pACYC184, grown for 5 h in MgM/MES at pH 5.0, and then lysed for co-immunoprecipitation. Membranes were probed with antibodies against T7 to detect $SsaQ_S$-T7 or HA to detect $SsaQ_L$-HA and $SsaQ_S$-HA. *B*, the C-terminal region of $SsaQ_L$ is required for interaction with $SsaQ_S$. Bacterial strain *ssaQ* p*ssa*Q_S*-T7* was cotransformed with p*ssaQ-HA* or p*ssa*Q_{LN216}*-HA* and used for co-immunoprecipitation. *C*, dimerization of $SsaQ_S$. *E. coli* BL21(DE3) cells containing pET-*ssa*Q_S were subjected to treatment with the cross-linker disuccinimidyl suberate (DSS) or DMSO and then analyzed by immunoblotting. *D*, the C-terminal region of $SsaQ_L$ is required for self-interaction. The *ssaQ* mutant (HH225) was transformed with the indicated plasmids and used for immunoprecipitating HA-tagged protein. Samples were analyzed by immunoblotting. *E*, stabilization of $SsaQ_L$ requires $SsaQ_S$. The *ssaQ* gene in the wild-type strain was replaced with *ssa*Q_{M217L}*-HA* to create the *ssa*Q_L*-HA* strain (HH232) and transformed with p*ssa*Q_S or vector pWSK29 for analysis. Transformants were cultured for 4 h in MgM/MES at pH 5.0, and tetracycline was added to stop protein synthesis. Samples were taken at the indicated time points for analysis. The band intensities were measured with Image J software and normalized with DnaK to construct the stability curve of $SsaQ_L$-HA.

minimal medium at pH 5.0 to express $SsaQ_L$-HA and other SPI-2 proteins. Tetracycline was then added to the growth medium to stop protein synthesis, and samples were taken at different time points to analyze protein stability by SDS-PAGE and immunoblotting. As shown in Fig. 4*E*, $SsaQ_L$ was markedly less stable in the absence of $SsaQ_S$.

Structural Predictions—The level of sequence similarity between $SsaQ_S$ and the flagellar C-ring protein FliN (29) is sufficient for a reliable homology model to be calculated. The template structure of FliN exists as an intimately intertwined dimer that forms a saddle shape (Fig. 5). The homology model of an $SsaQ_S$ monomer shows the expected extended β-sheet structure and displays significant solvent exposure of hydrophobic residues, suggesting a higher order organization. The ability of $SsaQ_S$ to oligomerize in solution was confirmed by ^{1}H NMR spectroscopy (data not shown), in which concentration-independent line-widths of $SsaQ_S$ were consistent with a molecular species between ~20 and ~40 kDa. These data indicate that

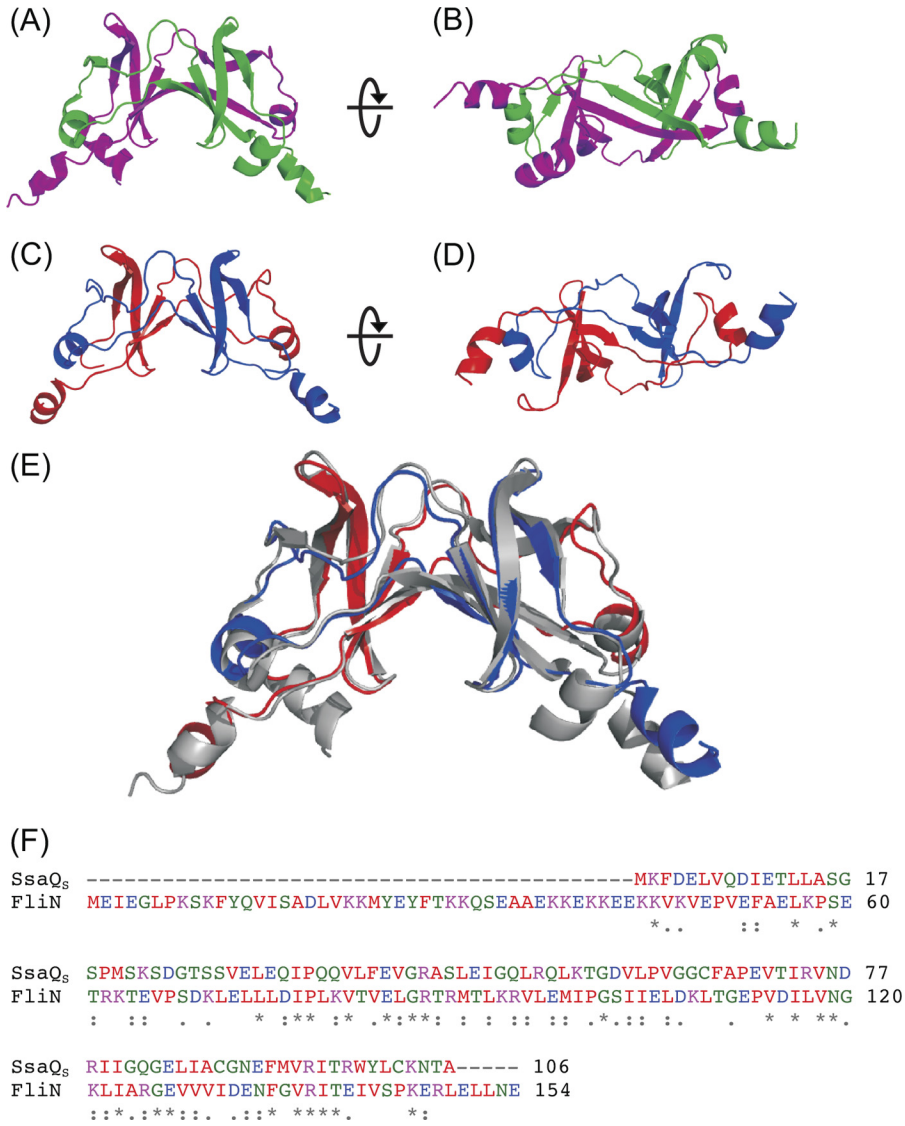

FIGURE 5. **Alignment and three-dimensional structure of $SsaQ_S$ and FliN.** *A* and *B*, two orthogonal views of the ribbon representation of the flagellar rotor protein FliN from *T. maritima* (Protein Data Bank code 1YAB). Individual monomers are shown in *pink* and *green*. *C* and *D*, two orthogonal views of the homology model of the $SsaQ_S$ dimer. Individual monomers are shown in *blue* and *red*. *E*, superposition of the FliN (*gray*) and $SsaQ_S$ (*blue*/*red*) dimer structures. *F*, sequence alignment of $SsaQ_S$ and FliN.

$SsaQ_S$ probably exists as the intertwined dimer as observed in the crystal structure of FliN.

$SsaQ_S$ Contributes to sv. Typhimurium Virulence—Because $SsaQ_S$ is required to stabilize $SsaQ_L$ and because $SsaQ_L$ is an essential component of the SPI-2 T3SS, we tested if $SsaQ_S$ contributes to virulence by CI analysis (30) involving mixed infections of the wild-type and *ssa*Q_S mutant strains in the sv. Typhimurium/mouse model of systemic disease. Approximately 5×10^2 colony-forming units of each strain were combined and used to inoculate BALB/c mice by the intraperitoneal route. Infection was allowed to proceed for 4 days, at which time mice were killed, and spleens were homogenized and plated onto rich medium to determine the colony-forming units of each strain. The resulting CI was 0.206 ± 0.133, indicating that the *ssa*Q_S mutant strain is attenuated in virulence. The virulence defect was fully complemented by introducing p*ssa*Q_S into the mutant strain and determining its CI in relation to the wild-type strain (CI 0.910 ± 0.212, $p = 0.0013$) and partially complemented by introducing p*ssa*Q_L into the mutant strain (CI of 0.448 ± 0.067, $p = 0.0171$). This showed that $SsaQ_S$ contributes to sv. Typhimurium virulence in the mouse model of infection. The partial complementation of virulence by overexpression of $SsaQ_L$ in the *ssa*Q_S mutant suggested either that $SsaQ_S$ has an additional function or that overexpressing $SsaQ_L$ somehow affects the virulence of sv. Typhimurium. To test this, we measured the virulence of the *ssa*Q_L mutant carrying p*ssa*Q_L compared with the wild-type strain in mice. The resulting CI was 0.317 ± 0.10, significantly lower than 1 ($p = 0.0008$) but significantly higher than the CI obtained from the *ssa*Q_L mutant strain mixed with the wild-type strain (0.013 ± 0.005, $p = 0.0009$). This indicates that overexpressing $SsaQ_L$ in sv. Typhimurium affects its virulence in the mouse model of infection.

DISCUSSION

In this work, we discovered that the *ssaQ* gene of sv. Typhimurium SPI-2 encodes two proteins: the predicted full-length

protein $SsaQ_L$, composed of 322 amino acids, and the shorter protein $SsaQ_S$, comprising the C-terminal 106 residues of $SsaQ_L$. $SsaQ_L$ is essential for SPI-2 T3SS function, whereas $SsaQ_S$ stabilizes $SsaQ_L$ and augments the activity of the T3SS.

Site-directed mutagenesis and promoter analysis revealed that $SsaQ_S$ is a tandem translated product of *ssaQ*. Tandem translation or "in-frame initiated translation" often occurs in viruses and bacteriophage, where DNA coding capacity is limited, but has rarely been found in bacteria. The few cases reported to date include the widely conserved translational initiation factor IF2 (34), the chemotactic signaling protein CheA (35), the heat shock protein ClpB (36), and the methylation-dependent endonuclease component McrB from *E. coli* (37). It is also possible that the flagellar secretion system component FliO from *Salmonella* is tandemly translated, but this has not yet been shown in wild-type cells (38). To our knowledge, $SsaQ_S$ represents the first example of a bacterial virulence factor produced by tandem translation and of a chaperone produced by this process.

The finding that $SsaQ_L$ is required for secretion of translocon proteins and effectors is not surprising given its sequence similarity to C-ring components of other bacteria. Homologs of $SsaQ_L$, including Spa33 from *Shigella* and YscQ from *Yersinia*, are C-ring proteins that interact with components of the secretion machinery, including the ATPase and its regulators (16, 39), and are necessary for formation of the needle structure of the T3SS (16) and assembly of the associated complex between ATPase and the C-ring (17). We have also observed that $SsaQ_L$ interacts with the putative SPI-2 T3SS ATPase SsaN.[3] Therefore, $SsaQ_L$ is very likely to be an essential C-ring component of the SPI-2 secretion machinery.

In the flagellar C-ring, FliN interacts with the C-terminal region of FliM ($FliM_{248-334}$), where the two proteins show sequence similarity (40). A similar situation is found in the related $HrcQ_B$ and $HrcQ_A$ proteins of the *Pseudomonas syringae* pv. phaseolicola T3SS (41). Here, the C-terminal region of the smaller $HrcQ_B$ protein interacts with the C-terminal region of $HrcQ_A$. However, the precise role of $HrcQ_B$ in the function of the *P. syringae* T3SS is not clear. Structural studies have revealed that FliN forms a tetramer that links molecules of FliM to create a large repeating structure that comprises the lower region of the flagellar C-ring (42). FliN is thus an integral component of the C-ring. The obvious similarities between $SsaQ_S$ and FliN (Fig. 5) and our finding that $SsaQ_S$ interacts with an identical region in a larger protein that is predicted to be part of the SPI-2 T3SS C-ring suggested that $SsaQ_S$ is also a C-ring protein that could act as a bridge between molecules of $SsaQ_L$ to form a repeating unit at the bottom of the C-ring (42). Although this remains a possibility, two results suggest otherwise. First, it is clear that the SPI-2 T3SS is partially functional in the absence of $SsaQ_S$ and that the secretion and translocation defects can be completely overcome *in vitro* and in infected cells by overexpression of $SsaQ_L$ (Figs. 2 and 3). This contrasts with the situation in the flagellar system, where FliN has an essential role in C-ring formation and flagellum assembly (43, 44). Second, we have shown that $SsaQ_L$ can oligomerize in the absence of $SsaQ_S$. Instead, our data show that $SsaQ_S$ has a chaperoning function by binding to its corresponding region within $SsaQ_L$, stabilizing the larger protein. Consistent with this, $SsaQ_S$ has a predicted molecular mass of 11.7 kDa and an acidic pI (4.7), features that typify many chaperones (45). It is possible that $SsaQ_L$ and $SsaQ_S$ heterodimerize, in which case $SsaQ_S$ would assist in the proper folding of $SsaQ_L$ or prevent self-polymerization or degradation of $SsaQ_L$ before it docks to the T3SS apparatus. Once there, a conformational change induced by interaction with component(s) of the apparatus might lead to the dissociation of the heterodimer. In another scenario, a tetramer could be constructed from $SsaQ_S$ homodimers interacting with $SsaQ_L$ dimers. The fact that the two SsaQ variants are cotranslated from the same transcript would favor the formation of a heterodimer, as the two proximal polypeptides would fold and dimerize together as they simultaneously exited from ribosomes. It is conceivable that, during assembly of higher order structures, $SsaQ_L$ then displaces $SsaQ_S$ to construct the mature active SPI-2 T3SS.

To our knowledge, $SsaQ_S$ is the first example of a chaperone for a C-ring protein. It is interesting to note that, in the T3SSs encoded by *Salmonella* SPI-1, *Shigella*, and *Yersinia*, there is no evidence for the presence for a small C-ring protein that would correspond to FliN or $SsaQ_S$, suggesting that the C-rings of these T3SSs could assemble through direct oligomerization of their $SsaQ_L$ homologs (SpaO, Spa33, and YscQ, respectively). Structural studies on $SsaQ_L$ are now required to reveal the architecture and to help understand the function of the putative C-ring of the SPI-2 T3SS.

[3] X.-J. Yu, P. J. Simpson, M. Liu, S. Matthews, and D. W. Holden, unpublished data.

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