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

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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 (SsaQL) composed of 322 amino acids and a shorter protein (SsaQS) comprising the C-terminal 106 residues of SsaQL. SsaQS is essential for SPI-2 T3SS function. Loss of SsaQS impairs the function of the T3SS both ex vivo and in vivo. SsaQS binds to its corresponding region within SsaQL and stabilizes the larger protein. Therefore, SsaQS 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⁺, Kan⁺, Cam⁺, and Tetr, 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 ssaQS point
TABLE 1

| sv. Typhimurium strains constructed and used in this study | Description | Source or Ref. |
|-----------------------------------------------------------|-------------|----------------|
| 12023 Wild-type                                           | NCTC        |
| P3F4 ssaM::In5 in 12023 (Kan')                             | Ref. 9      |
| HH216 ssa-2HA::cat in 12023 (Cam')                         | Ref. 21     |
| HH225 ΔssaQ_{NatG413}::Kan in 12023 (Kan')                | This study  |
| HH226 ssa-2HA::cat in ΔssaQ_{NatG413}::Kan mutant          | This study  |
| HH227 ΔssaQ_{NatG413}::cat in 12023 (Cam')                 | This study  |
| HH228 ssaQ replaced with ssaQ_{NatG413} in 12023           | This study  |
| HH229 ssaQ replaced with ssaQ_{NatG413} in 12023           | This study  |
| HH230 ssa-2HA::cat in H9262                                 | This study  |
| HH231 ssa-2HA::cat in H9262                                 | This study  |
| HH232 ssaQ replaced with ssaQ_{NatG413}::HA::Kan in 12023 | This study  |

mutant HH228 or ssaQ, point mutant HH229. Briefly, ssaQ was ligated into pCR2.1-TOPO (Invitrogen) using primers ssaQf and ssaQHAb and ligated into pCR-ssaQ. Plasmids ssaQ and ssaQ_{NatG413} were constructed by subcloning ssaQ and ssaQ_{NatG413} into pCR-ssaQ. The ssaQ_{NatG413} gene was amplified using primers ssaQf and ssaQHAb and ligated into pCR-ssaQ. The HA-tagged ssaQ, ssaQ_{NatG413}, and ssaQ_{NatG413} DNA fragments were amplified from pCR-ssaQ, pCR-ssaQ_{NatG413} and pCR-ssaQ_{NatG413} using primers ssaQf and ssaQHAb, and ligated into pCR-ssaQ to create plasmids pssaQ-HA, pssaQ_{NatG413}-HA, and pssaQ_{NatG413}-HA. The ssaQ_{NatG413} fragment was amplified using primers ssaQf and ssaQHAb, and ligated into pCR-ssaQ to create plasmids pssaQ-HA, pssaQ_{NatG413}-HA, and pssaQ_{NatG413}-HA. The ssaQ_{NatG413} fragment was amplified using primers ssaQf and ssaQHAb, and ligated into pCR-ssaQ to create plasmids pssaQ-HA, pssaQ_{NatG413}-HA, and pssaQ_{NatG413}-HA. The HA-Q fragment was amplified using primers ssaQf and ssaQHAb, and ligated into pCR-ssaQ to create plasmids pssaQ-HA, pssaQ_{NatG413}-HA, and pssaQ_{NatG413}-HA. The ssaQ_{NatG413} fragment was amplified using primers ssaQf and ssaQHAb, and ligated into pCR-ssaQ to create plasmids pssQA::HA, pssQA::HA/ssaQ_{NatG413}-T7, and pssQA::HA/ssaQ_{NatG413}-T7, respectively. Plasmid pssQA::T7 was constructed by replacing the cat gene of pACYC184 (24) with ssaQ_{NatG413} following PCR amplification with primers ssaQf and ssaQHAb. The expression of ssaQ_{NatG413} 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 (using primers 102saaM-KpnI and ssaQ_{NatG413}-T7-XbaI) or beginning from the start codon of ssaM to the start codon of ssaQ (using primers ssaM-KpnI and ssaQ_{NatG413}-T7-XbaI), amplified from genomic DNA of sv. Typhimurium strain 12023, were ligated into the KpnI and XbaI sites of pPV25, a vector carrying promoterless gfpmut3A (25), to create p102saaM::MQ_{NatG413} and pssaq-{NatG413} respectively. To express ssaQ_{NatG413} with a His6 tag, the cleaved ssaQ_{NatG413} PCR product (using primers ssaQ_{NatG413}-EcoRI and ssaQ_{NatG413}-XhoI) was ligated into pET22b (Novagen) to construct pET-ssaQ_{NatG413}. Plasmids constructed as part of this study were verified by DNA sequencing and are listed in Table 2. Plasmid pstein-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 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 pre-clear the lysate. The pre-cleared 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 SsaQs—The bacterial lysate of ssaQ pssaQ-HA culture grown in MgM/MES was subjected to immunoprecipitation with rat anti-HA antibody. SsaQs-HA was recovered from the PVDF membrane and sequenced by...
Edman degradation (Protein & Nucleic Acid Chemistry Facility, University of Cambridge). The first six amino acids obtained for SsaQs were MKFDEL.

**Cross-linking and Protein Stability Assays**—Escherichia coli BL21(DE3) cells containing plasmid pET-ssaQs were cultured in LB medium and induced for 2 h at 30 °C with 1 mM isopropyl β-D-thiogalactopyranoside to express SsaQs–His6. The bacteria were treated with DMSO (Sigma) or the cross-linker disuccinimidyldiethylenetriamine (1 mM; Pierce) as recommended by the manufacturer.

**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 (Millipore) membranes and examined using the ECL detection system (Amersham Biosciences) under conditions recommended by the manufacturer. The membranes were then incubated with primary antibodies, followed by incubation with HRP-conjugated anti-His antibody (ab1187, Abcam).

**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).

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

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. 1B). However, under these conditions, SsaQ-HA was not detected in culture supernatants or on the bacterial cell surface (Fig. 1B). Interestingly, two proteins were detected in the bac-

**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 pssaQ-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-L-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.
Tandem Translation for Salmonella SsaQ

The expression of ssaQ is controlled by a promoter of the 5.4-kb ssaMVNOPQ operon (13, 14). To determine whether SsaQ 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 ssaQS were ligated into a gfp reporter plasmid to generate plasmids p102ssaMQLN217 and psma-MQ_LN217, respectively (Fig. 1C). 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. 1C) (32). These results indicate that both SsaQ and SsaQS are translated from the ssaMVNOPQ transcript.

To determine whether SsaQS is a cleavage product of SsaQ, 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 null mutant strain grown in minimal medium at pH 5.0. If SsaQS is the product of cleavage of SsaQ, then it should not be produced if SsaQ is truncated before Met-217. Therefore, the codon for Tyr-206 of SsaQ-HA was replaced with a stop codon (ochre mutation, TAA). As expected, SsaQ_HA was not detected upon expression of SsaQY206oc-HA, but SsaQ-HA was still produced by this mutant (Fig. 1D). Substituting the codon for Met-217 (the putative start codon for SsaQ) with a leucine codon (SsaQM217L) resulted in an absence of SsaQS-HA but not SsaQ_HA (Fig. 1D). These results show that SsaQ is not a product of cleavage of SsaQ, and that SsaQ and SsaQS are translated independently from the same mRNA species. Consistent with this, there is a purine-rich sequence upstream of the start codon of ssaQ (AGAG-GATAACACGACATG), which is likely to be the Shine-Dalgarno sequence for translational initiation (33).

Next we used these strains to examine the involvement of SsaQ and SsaQS in SPI-2 T3SS function. In the absence of SsaQ, (the ssaQ null mutant strain expressing SsaQY206oc-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. 1E). However, the absence of SsaQ (the ssaQ null mutant strain expressing SsaQM217L-HA from a plasmid) did not noticeably affect secretion of SseB or SseJ at pH 5.0 (Fig. 1E). This result indicates that SsaQ is functional and that SsaQ is essential for the SPI-2 T3SS, whereas SsaQS is not.

SsaQ Is Required for Efficient Secretion of Translocon and Effector Proteins—To further investigate the possible function of SsaQS, the point mutation for M217L was introduced into the chromosomal ssaQ gene to create a mutant in which SsaQ was produced as a result of expression from chromosomal DNA but in which SsaQS 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. 2A). The decreased secretion of these proteins in the ssaQ mutant was restored to wild-type levels by introduction of a plasmid expressing either ssaQS or ssaQ, (Fig. 2A). The ability of overexpressed SsaQ to compensate for loss of SsaQS was also evident when ssaQ was overexpressed in the ssaQ mutant (Fig. 1E). These results indicate that SsaQS is required for the efficient secretion of translo-

![Image](350x338 to 491x352)

![Image](350x354 to 491x383)

![Image](350x445 to 491x457)

![Image](350x481 to 491x494)

![Image](350x589 to 477x607)

![Image](350x610 to 477x628)

![Image](350x631 to 477x649)

![Image](350x652 to 477x669)

FIGURE 2. Role of SsaQS in secretion of translocon and effector proteins. The ssaQ gene of the wild-type strain of sv. Typhimurium was replaced with ssaQY206oc or ssaQM217L to create the ssaQS (HH229) or ssaQ (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 psteC-2HA were used for pH shift analysis. Secretion of translocon protein SseB was used as an additional control.
con proteins and that overexpression of SsaQ_L can compensate for the loss of SsaQS

To examine the potential role of SsaQS 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 ssaQS mutant strains complemented with either ssaQL or ssaQS displayed greatly enhanced and similar levels of SseJ-2HA secretion, respectively (Fig. 2B). In contrast, quantification of the amount of SseJ-2HA secreted by the ssaqs mutant showed that it was ~44% of the wild-type level (Fig. 2B). Mutation of ssaQ_L caused a similar reduction in the secreted levels of another effector, SteC-2HA (Fig. 2C). These findings indicate that the translational-to-effector switch mediated by the SsrA-SpiC-SsaM complex in response to pH shift (4) still occurs in the absence of SsaQS, but the overall efficiency of secretion through the SPI-2 T3SS is reduced in vitro.

**SsaQS Is Required for Efficient Translocation of Effectors**—
We next examined whether SsaQS 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_L mutant bearing the complementing plasmid pssaQ_L or pssaQ_S 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_L mutant showed that it was ~50% of the wild-type level. We concluded that SsaQ_L is required for efficient translocation of SPI-2 T3SS effectors in infected host cells.

**SsaQS Interacts with and Stabilizes SsaQ_L**—
The observation that overexpression of SsaQ_L compensates for the loss of SsaQS suggested that SsaQ_L might function as a chaperone, stabilizing SsaQ_L. To test if SsaQ_L interacts with SsaQ_S, the ssaQ_L mutant strain containing plasmid pssaQ-L-HA (which produces both SsaQ_L-HA and SsaQ_S-HA) and either pssaQ_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_L-T7. SsaQ_L-HA and SsaQ_S-HA were not co-immunoprecipitated in the negative control strain containing plasmids pssaQ-HA and pACYC184. However, both SsaQ_L-HA and SsaQ_S-HA were co-immunoprecipitated from the strain containing pssaQ-L-HA and pssaQ_S-T7 (Fig. 4A). This result suggested that SsaQS 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_L mutant containing plasmid pssaQ_L-HA, 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 pssaQ-L-HA and pssaQ_S-T7 (Fig. 4B). However, the antibody did not immunoprecipitate SsaQ_S-T7 in the ssaQ_L mutant containing pssaQ_LN216-HA and pssaQ_S-T7 (Fig. 4B). In addition, a cross-linking experiment showed that SsaQS homodimerizes following its production in *E. coli* (Fig. 4C). 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_S-HA or SsaQ_LN216-HA were transformed into the ssaQ_L 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. 4D). Therefore, the SsaQ_S region of SsaQ_L, but not SsaQ_L 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_M2171-HA but not SsaQ_L. This strain was then transformed with pssaQ_S or the empty vector (pWSK29). The transformants were grown in...
minimal medium at pH 5.0 to express SsaQL-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. 4E, SsaQL was markedly less stable in the absence of SsaQS.

### Structural Predictions

The level of sequence similarity between SsaQS 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 SsaQS monomer shows the expected extended β-sheet structure and displays significant solvent exposure of hydrophobic residues, suggesting a higher order organization. The ability of SsaQS to oligomerize in solution was confirmed by 1H NMR spectroscopy (data not shown), in which concentration-independent line-widths of SsaQS were consistent with a molecular species between ~20 and ~40 kDa. These data indicate that SsaQS functions as a chaperone for SsaQL.

**FIGURE 4. SsaQS functions as a chaperone for SsaQL.** A, SsaQS interacts with SsaQL and itself. Bacterial strain ssaQ pssaQ-HA was cotransformed with pssaQL-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 SsaQL-T7 or HA to detect SsaQL-HA and SsaQS-HA. B, the C-terminal region of SsaQL is required for interaction with SsaQS. Bacterial strain ssaQ pssaQ-T7 was cotransformed with pssaQ-HA or pssaQL216-HA and used for co-immunoprecipitation. C, dimerization of SsaQS. E. coli BL21(DE3) cells containing pET-ssaQS were subjected to treatment with the cross-linker disuccinimidyl suberate (DSS) or DMSO and then analyzed by immunoblotting. D, the C-terminal region of SsaQL 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 SsaQL requires SsaQS. The ssaQ gene in the wild-type strain was replaced with pssaQM217L-HA to create the ssaQL-HA strain (HH232) and transformed with pssaQS 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 SsaQL-HA.
SsaQS probably exists as the intertwined dimer as observed in the crystal structure of FliN.

**SsaQS Contributes to sv. Typhimurium Virulence**—Because SsaQS is required to stabilize SsaQL and because SsaQL is an essential component of the SPI-2 T3SS, we tested if SsaQS contributes to virulence by CI analysis involving mixed infections of the wild-type and ssaQS mutant strains in the sv. Typhimurium/mouse model of systemic disease. Approximately 5 × 10⁵ 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 ssaQS mutant strain is attenuated in virulence. The virulence defect was fully complemented by introducing pssaQS into the mutant strain and determining its CI in relation to the wild-type strain (CI of 0.448 ± 0.067, p = 0.0171). This showed that SsaQS contributes to sv. Typhimurium virulence in the mouse model of infection. The partial complementation of virulence by overexpression of SsaQL in the ssaQS mutant suggested either that SsaQS has an additional function or that overexpressing SsaQL somehow affects the virulence of sv. Typhimurium. To test this, we measured the virulence of the ssaQL mutant carrying pssaQL compared with the wild-type strain in mice. The resulting CI was 0.317 ± 0.10, significantly lower than 1 (p = 0.008) but significantly higher than the CI obtained from the ssaQL mutant strain mixed with the wild-type strain (0.013 ± 0.005, p = 0.0009). This indicates that overexpressing SsaQS 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...
Tandem Translation for Salmonella SsaQ

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

Site-directed mutagenesis and promoter analysis revealed that SsaQS 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 ClpP (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, SsaQS represents the first example of a bacterial virulence factor produced by tandem translation and of a chaperone produced by this process.

The finding that SsaQL 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 SsaQL, 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 SsaQL interacts with the putative SPI-2 T3SS ATPase SsaN. Therefore, SsaQL 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<sub>248–334</sub>), where the two proteins show sequence similarity (40). A similar situation is found in the related HrcQB and HrcQ<sub>α</sub> proteins of the *Pseudomonas syringae* pv. *phaseolicola* T3SS (41). Here, the C-terminal region of the smaller HrcQ<sub>α</sub> protein interacts with the C-terminal region of HrcQB. However, the precise role of HrcQ<sub>α</sub> 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 SsaQS and FliN (Fig. 5) and our finding that SsaQS interacts with an identical region in a larger protein that is predicted to be part of the SPI-2 T3SS C-ring suggested that SsaQS<sub>L</sub> is also a C-ring protein that could act as a bridge between molecules of SsaQL 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 SsaQS<sub>L</sub> and that the secretion and translocation defects can be completely overcome in *vivo* and in infected cells by overexpression of SsaQL (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 SsaQL can oligomerize in the absence of SsaQS<sub>L</sub>. Instead, our data show that SsaQS<sub>L</sub> has a chaperoning function by binding to its corresponding region within SsaQL, stabilizing the larger protein. Consistent with this, SsaQL 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 SsaQL and SsaQS<sub>L</sub> heterodimerize, in which case SsaQS<sub>L</sub> would assist in the proper folding of SsaQL or prevent self-polymerization or degradation of SsaQL 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 SsaQS homodimers interacting with SsaQL 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, SsaQL then replaces SsaQS<sub>L</sub> to construct the mature active SPI-2 T3SS.

To our knowledge, SsaQL 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 SsaQS, suggesting that the C-rings of these T3SSs could assemble through direct oligomerization of their SsaQ homologs (SpaO, Spa33, and YscQ, respectively). Structural studies on SsaQL are now required to reveal the architecture and to help understand the function of the putative C-ring of the SPI-2 T3SS.

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