Shigella Spa33 Is an Essential C-ring Component of Type III Secretion Machinery*

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Type III secretion machinery (TTSM), composed of a needle, a basal body, and a C-ring compartment, delivers a subset of effectors into host cells. Here, we show that Shigella Spa33 is an essential component of the C-ring compartment involved in mediating the transit of various TTSM-associated translocated proteins. Electron microscopic analysis and pull-down assay revealed Spa33 to be localized beneath the TTSM via interaction with MxiG and MxiJ (basal body components). Spa33 is also capable of interacting with Spa47 (TTSM ATPase), MxiK, MxiN (required for the transit of MxiH, the needle component), Spa32 (required for determining needle length), and several effectors. Genetic and functional analyses of the Spa33 C-terminal region, which is highly conserved in the SpaO-YscQ-HrcQB-FliN family, indicate that some of the conserved residues are crucial for needle formation via interactions with MxiN. Thus, Spa33 plays a central role as the C-ring component in recruiting/exporting TTSM-associated proteins.

Type III secretion machinery (TTSM) is found in many Gram-negative bacteria and plays a central role in mediating the translocation of virulence proteins (effectors) into host cells. TTSM consists of three major components: a needle portion, a basal body and a C-ring compartment, and the entire complex is needed to provide a conduit for direct translocation of effectors from the bacterial cytoplasm into host cells (1–3). Structural studies of the TTSM of Salmonella and Shigella indicate that the basal body resembles the flagella basal body, which consists of two upper and two lower rings (4–8). The compartment called the C-ring, assembled beneath the flagella basal body, is also present beneath the TTSM basal body. However, its relevant component and exact role in protein translocation via the TTSM remain largely speculative (9–12). The needle portion of the Shigella TTSM is composed of MxiH and MxiI, and the basal body is composed of MxiD, MxiG, and MxiJ (8, 13). Genetic and functional analyses indicate that some of the TTSM-associated proteins such as MxiK, MxiN, Spa32, and Spa47 are involved in forming the functional needle, where MxiN is thought to be required for translocation of MxiH, the major needle component, through the basal body (14–19). The C-ring compartment of TTSM is assumed to be involved in recognition of the secreted signals carried by effectors and to be responsible for the promiscuous character of the TTSM in regards to heterologous secreted proteins. However, the mechanisms underlying the recognition and export of proteins via the TTSM components remain essentially unknown.

Previous studies have indicated that Pseudomonas syringae HrcQ was located on the cytoplasmic side of the TTSM. HrcQ shares partial amino acid sequence similarity with FilI (a flagella C-ring component), Shigella Spa33, Salmonella SpaO, and Yersinia YscQ (12, 20, 21). We therefore sought to characterize the role of Spa33 in mediating the translocation of Shigella effectors via the TTSM. Our results provide the first direct evidence that, in the TTSM of animal pathogens, Spa33 is an essential C-ring component required for TTSM needle formation.

EXPERIMENTAL PROCEDURES

Bacteria and Plasmids

The bacterial strains and plasmids used are listed in supplemental Table S1. The various spa33 gene fragments amplified by PCR were digested with BamHI and SalI and then ligated into the corresponding sites of pTB-Myc or pGST. The point mutants on pMW-spa33 and pGST-spa33 were created with a QuikChange site-directed mutagenesis kit (Stratagene). All S. flexneri-derived strains were grown routinely in brain heart infusion (BHI) broth (Difco, Detroit, MI) or L-broth. Strains harboring pMW119-Tp, pTB101-Tp, or pCACTUS-Tp-based plasmids were grown in Mueller-Hinton broth (Difco) supplemented with 5 μg/ml trimethoprim.

Construction of a Nonpolar spa33 Mutant of S. flexneri

For construction of a nonpolar spa33 mutant, the aphA-3 (kanamycin resistance gene) cassette specifically designed for the construction of a nonpolar mutant was used (22).

Production and Preparation of Recombinant Proteins

For GST-fused proteins, Escherichia coli BL21 harboring pGEX-4T-1derivatives were cultivated in L-broth supplemented with ampicillin (50 μg/ml) for 3 h at 37 °C. Expression was induced by the addition of 1 or 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubation for 3 h at 37 °C. Bacteria were disrupted by sonication. Purification of the GST-fused proteins with glutathione-Sepharose 4B beads (Amersham Biosciences) was performed according to the manufacturer’s protocol.
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Antibodies

Anti-IpaB, -IpaC, and -IpaD antibodies used for immunnoblotting were described previously (23). Anti-MxiD, -MxiG, -MxiI, and -Spa32 antibodies for immunnoblotting were also described previously (8, 19). The rabbit anti-Spa33 and -Spa47 antibodies were raised against recombinant Spa33 and Spa47 proteins. The GST proteins were cleaved with thrombin according to the manufacturer’s protocol. Anti-MxiK and -MxiN antibodies were prepared as follows. The synthetic peptides of MxiK-(CNEGMQYAKRHTFGIOTSL) and MxiN-(CRQIAEFLK-KNPVND) were conjugated to keyhole limpet hemocyanin (keyhole limpet hemocyanin) and injected into rabbits. Anti-FLAG antibody (Sigma) and anti-Myc antibody (Santa Cruz Biotechnology) were obtained commercially.

Analysis of Proteins Secreted by S. flexneri

A small aliquot of an overnight culture of S. flexneri grown at 30 °C in L-broth was inoculated into 20 ml of BHI broth, and the bacteria were grown at 37 °C for 2.5 h to an optical density of 1.3 at 600 nm. Proteins obtained commercially.

Isolation of the Type III Needle Complexes from S. flexneri

A 10-ml aliquot from an overnight culture grown at 30 °C in L-broth was inoculated into 1 liter of L-broth, and the bacteria were grown until the late log phase (1–2 h) to an optical density of 1.1. IPTG was then added to a final concentration of 0.1 mM. After incubation for 2 h at 37 °C, bacteria were harvested. The bacterial culture was collected and lysed by sonication in 1 ml of ice-cold RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM AEBSF, pH 7.5). The lysates were centrifuged at 100,000 × g for 10 min at 4 °C, and the supernatants (lysates) were used for pull-down assay.

Binding Analysis by GST Pull-down Assay

The GST-fused protein derivatives bound to 25 µl of glutathione-Sepharose 4B beads were mixed with a cleared extract of MC1061 harboring pTB101-Tp or pTB-Myc and incubated for 2 h at 4 °C. Supernatants were removed by centrifugation, and beads were washed four times with ice-cold RIPA buffer. After the final wash, supernatants were removed, and 25 µl of SDS-PAGE sample buffer were added to each sample.

RESULTS

Spa33 Is Essential for TTSM Needle Formation—To characterize the role of Spa33 in TTSM function, a non-polar spa33 deletion mutant (Δspa33) (Fig. 1A) in YSH6000 (wild-type S. flexneri) was investigated for its capacity to secrete IpaB, IpaC, and IpaD. In agreement with a previous study (25), we confirmed that the Ipa proteins were secreted into phosphate-buffered saline containing 0.003% Congo red (CR supernatant), a conditional medium that stimulates TTSM activity, by WT and Δspa33/pMW-spa33 but not by Δspa33 or del-17 (a TTSM-defective mutant) (Fig. 1B, top). Although Spa33 was present in whole cell lysates prepared from WT or Δspa33/pMW-spa33, none was detected in the CR supernatant (Fig. 1B, bottom). TEM analysis of the TTSM extensively purified from Δspa33 revealed that Δspa33 produces a defective TTSM without the needle portion (Fig. 1C, panel b), a defect similar to that of the mxiH mutant (defective in production of the TTSM needle component) or the spa47 mutant (ATPase defect) (8). When pMW-spa33 was introduced into Δspa33 (Δspa33/pMW-spa33), the capacity to produce intact TTSM was restored (Fig. 1C, panel c) to the WT level (Fig. 1C, panel a). In addition, TEM analysis of the osmotically shocked cells ensured TTSM with needles within the envelopes of WT and Δspa33/pMW-spa33 but not in the Δspa33 envelope (Fig. 1C, bottom), suggesting that spa33 is essential for the formation of TTSM needles.

Spa33 Is Located beneath the TTSM in the Cytoplasm—To determine the localization of Spa33, we investigated whether Spa33 was present in the purified TTSM from WT by immunoblotts with anti-Spa33, -MxiD, -MxiG, or -MxiI antibody. Spa33 was detected with MxiD, MxiG, and MxiI in the purified TTSM but not in the corresponding

Electron Microscopy

(i) Observation of Purified Type III Needle Complexes and Osmotically Shocked Cells of S. flexneri—Samples were placed onto carbon-coated copper grids and stained with rabbit anti-Myc antibody and Protein A conjugated with gold colloidal particles (5 and 10 nm diameter; EY Laboratory). Samples were stained with uranyl acetate and observed, then images were obtained with a JEM-1200EX TEM (JEOL).

(ii) Immunoelectron Microscopy—The post-embedding immunogold method was employed S. flexneri, cultured at 37 °C to an optical density of 1.0 at 600 nm, was harvested. The bacteria were fixed with 1% glutaraldehyde in 100 mM phosphate buffer, dehydrated with a graded ethanol series, and embedded in epoxy resin. Ultra-thin sections were placed onto nickel-coated copper grids and stained with rabbit anti-Myc antibody and Protein A conjugated with gold colloidal particles (5 and 10 nm diameter; JEOL, Tokyo, Japan).

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Sample from del-17 (Fig. 2A). To pursue this, we analyzed the location of Spa33 in Spa33/pMW-Myc-spa33 by immuno-gold EM with anti-Myc antibody, and the results showed that the gold particles were mostly localized within the cytoplasm in the vicinity of the bacterial membrane (Fig. 2B). Since the MxiG and MxiJ of Shigella are the major inner membrane positional TTSM components (4, 8), we hypothesized that Spa33 was associated with MxiG or MxiJ and tested the idea by creating GST-MxiG and -MxiJ and performing pull-down assays with lysates prepared from E. coli expressing Spa33. Spa33 was pulled down by GST-MxiG, and although the amount was less than that pulled down by GST-MxiJ, Spa33 was also pulled down by GST-MxiJ (Fig. 2C). We then created various truncated versions of Spa33 to analyze the domain of Spa33 involved in binding with MxiG and MxiJ. We found Spa33217–293, Spa3381–216, and Spa331–80 but not Spa331–86 or Spa33217–293, to be pulled down by GST-MxiG and GST-MxiJ, suggesting that MxiG and MxiJ associate with the middle portion of Spa33 (Fig. 2D). To directly demonstrate the location of Spa33 in TTSM using EM, we treated Spa33/pMW-Myc-spa33 with dithiobis(succinimidyl propionate), a cross-linking reagent, and purified TTSM from the bacteria. The TEM analysis indicated a macromolecular structure to be associated with the lower portion of the TTSM basal body structure (Fig. 2E, arrows). Indeed, immuno-gold EM revealed that gold particles were detectable beneath the basal body of the TTSM (Fig. 2E). Based on the results of this series of experiments, we concluded that Spa33 is a cytoplasmic TTSM component associated with the major inner membrane positional TTSM components MxiG and MxiJ.

Spa33 Interacts with TTSM-associated Proteins—Our and other previous studies (4, 8, 14) have shown the mxiH, mxiI, mxiK, mxiN, spa32, or spa47 mutants to be deficient in forming the needle structure, as was the case in this study of the spa33 mutant (this study). These earlier results prompted us to hypothesize that the defective Spa33 phenotype is attributable to the interaction between Spa33 and some of the proteins involved in needle formation. To test this hypothesis, we initially attempted to investigate the capacity of Spa33 to interact with the TTSS-associated proteins using a yeast two-hybrid system. However, screening with the bait Spa33 yielded no positive clones, suggesting the bait to not be functional in the yeast two-hybrid system. Indeed, the same problem was also reported previously by other investigators (14). Thus, we created GST-MxiH, -MxiI, -MxiK, -MxiN, -Spa32, and -Spa47 and performed GST pull-down assays using bacterial lysates prepared from E. coli expressing Spa33. The proteins that bound with each of the GST-fused proteins were analyzed by immunoblotting with anti-Spa33 antibody. Spa33 was pulled down by GST-MxiH, -MxiI, -MxiK, -Spa32, and -Spa47 but not by GST-MxiN or -MxiJ (Fig. 3A). To confirm the ability of Spa33 to interact with these proteins in Shigella, we created Spa33/pFlag-spa33 and Spa33/pTB-spa33 and performed immunoprecipitation with anti-FLAG antibody. The bound proteins were analyzed by immunoblotting with anti-MxiH, -MxiI, -MxiK, -MxiN, -Spa32, and -Spa47 antibodies. MxiN, Spa32, and Spa47 were precipitated in Spa33/pFlag-spa33 but not in Spa33/pTB-spa33 (Fig. 3B), suggesting that Spa33 is capable of interacting with multiple proteins involved in needle formation (8, 14, 16, 19). MxiK was barely detectable under these conditions (data not shown), since MxiK was nearly undetectable in both of the bacterial lysates upon immunoblotting with anti-MxiK antibody.

To identify the Spa33 domain(s) involved in interacting with MxiH, Spa32, or Spa47, we created a series of GST-Spa33 versions (Fig. 3C, lower part) and subjected the resulting GST-Spa331, -spa331–293, -spa33217–293, -spa3331–216, and -spa3331–293 to GST pull-down assays using bacterial lysates prepared from E. coli expressing MxiN, Spa32, or Spa47. MxiN and Spa32 interacted with the C-terminal portion of Spa33 encompassing residues 217–293, while Spa47 interacted with the N-terminal 80-amino acid portion (Fig. 3C, top). To test the possibility that Spa33 interacts with other translocating effectors, we used GST pull-down assays to investigate the capacity of Spa33 to interact with

FIGURE 1. A. A genomic organization of the mxi-spa operon and construction of a spa33::aphA-3 mutant (Δspa33). B, whole cell lysates (WCL) and Congo-red induced supernatant (CR sup.) from YSH6000 (WT), del-17 (a TTSM-defective mutant), Δspa33, and Δspa33/pMW-spa33 were analyzed by immunoblotting with anti-IpaB, IpaC, and IpaD (upper panel) or anti-Spa33, antibodies (lower panel). C, purified TTSM from Shigella strains were observed by TEM (upper panels). Osmotically shocked cells were negatively stained and also observed by TEM (lower panels). Scale bars, 50 nm.
FIGURE 2. A, the purified TTSM samples were subjected to SDS-PAGE followed by CBB staining (upper panel) and immunoblotting with anti-Spa33, MxiD, MxiG, or MxiJ antibodies (lower panels). B, Spa33 located in the vicinity of the bacterial inner membrane is demonstrated by immuno-gold EM (arrows). The loci indicated by the arrows are shown in the insets. Scale bars, 0.2 mm. C, interactions of Spa33 with GST-MxiG and -MxiJ in the pull-down assay were visualized by immunoblotting with anti-Spa33 antibody. D, domain analysis of Spa33 involved in binding with MxiG and MxiJ are shown by the pull-down assay (right) and a schematic representation of the Spa33 derivatives (left) are shown. E, Spa33 located beneath the basal body is demonstrated by immuno-gold EM. Scale bars, 50 nm.
effectors, such as VirA, IcsB, IpaC, and IpgB1. Each of the GST-fused proteins created were mixed with bacterial lysates prepared from *E. coli* expressing Spa33, and the bound proteins were analyzed by immunoblotting with anti-Spa33 antibody. Although the extent varied among proteins, Spa33 was associated with all of the effectors examined, but not with GST alone (Fig. 3D), indicating that Spa33 has affinities for multiple proteins.

**Functional Analysis of Spa33 C-terminal Portion**—The C-terminal portion of Spa33 is highly conserved in Spa33 homologues of various TTSMs. Notably, the 9 residues in Spa33, i.e. Leu-242, Asn-266, Gly-267, Leu-269, Gly-273, Val-276, Gly-283, and Ile-286, are strictly conserved among flagella FliN, *Salmonella* SpaO, and *Yersinia* YscQ, and 5 of the 9 residues are also conserved in *Pseudomonas* HrcQB (Fig. 4A). We therefore substituted other residues having opposite chemical characteristics for each of the 9 residues in Spa33. Each of the point mutants created in the *spa33* gene cloned into pMW-*spa33* were introduced into */H9004*, and the effect of the single amino acid substitution on TTSM needle formation and the ability to secrete Ipa proteins was investigated. The Spa33 variants designated Spa33G273S and Spa33I286Q produced defective TTSM lacking needles, and Spa33L242Q produced heterogeneous TTSM composed of defective and normal structures (Fig. 4B), suggesting that Leu-242, Gly-273, and Ile-286 are critical residues for the functioning of Spa33. To confirm this, the abilities of the single-amino acid-substituted variants to secrete IpaB, IpaC, and IpaD into the CR supernatant were investigated. Like Δspa33, the Spa33(L242Q), Spa33(I286Q) and Spa33(L242Q, I286Q) variants were unable to secrete Ipa proteins into the CR supernatant (Fig. 4C). Since Spa33 has been shown to be translocated via the TTSM into the medium to determine needle length (16, 19), the effects of the single-amino acid-substituted variants of Spa33 on Spa32 secretion were also investigated. Spa33(L242Q), Spa33(G273S) and Spa33(L242Q, I286Q) but not the others, failed to secrete Spa32 (Fig. 4C), suggesting Leu-242, Gly-273, and Ile-286 in the C-terminal portion of Spa33 to be the critical residues for needle formation and effector secretion.

Since the C-terminal portion of Spa33 interacted with MxiN and Spa32, which are thought to be involved in needle formation (Fig. 3C) and length determination, respectively (14, 16, 19), we investigated Spa33(L242Q), Spa33(G273S) and Spa33(L242Q, I286Q) for their capacities to interact with MxiN, Spa32, or Spa47 (as the positive control). GST-Spa33(L242Q), -Spa33(G273S) and -Spa33(L242Q, I286Q) but not the others, failed to associate with MxiN, Spa32, or Spa47 under these conditions, but the amount was much smaller than that pulled down by critical residues for the functioning of Spa33. To confirm this, the abilities of the single-amino acid-substituted variants to secrete IpaB, IpaC, and IpaD into the CR supernatant were investigated. Like Δspa33, the Spa33(G273S), Spa33(I286Q) and Spa33(L242Q, I286Q) variants were unable to secrete Ipa proteins into the CR supernatant (Fig. 4C). Since Spa33 has been shown to be translocated via the TTSM into the medium to determine needle length (16, 19), the effects of the single-amino acid-substituted variants of Spa33 on Spa32 secretion were also investigated. Spa33(L242Q), Spa33(G273S) and Spa33(L242Q, I286Q) but not the others, failed to secrete Spa32 (Fig. 4C), suggesting Leu-242, Gly-273, and Ile-286 in the C-terminal portion of Spa33 to be the critical residues for needle formation and effector secretion.
These results thus suggest that the residues of Spa33 strictly conserved among its homologues play critical roles in interacting with MxiN, which has been indicated to mediate the transit of MxiH, the needle component (8, 13).

**Specificity of Spa33 Function to Shigella**—We investigated the capacities of the Spa33 homologues SpaO, YscQ, and HrcQB to interact with MxiN and other *Shigella*-TTSM-associated proteins, i.e. MxiK, Spa32, Spa47, MxiG, and MxiJ, by GST-pull down assay. SpaO was pulled down by GST-Spa33 WT (Fig. 4D). These results thus suggest that the residues of Spa33 strictly conserved among its homologues play critical roles in interacting with MxiN, which has been indicated to mediate the transit of MxiH, the needle component (8, 13).
all but GST-MxiG, while YscQ and HrcQB were not pulled down by any of the GST-fused proteins (Fig. 5A). This suggests that the inability of SpaO to interact with MxiG, or of YscQ and HrcQB to interact with TTSM components, may result in an inability to rescue the deficient phenotype of Δspa33. To confirm this, we investigated whether SpaO, YscQ, or HrcQB could rescue Spa33 function by introducing spaO, yscQ, and hrcQB clones, respectively, into Δspa33. We then employed immunoblotting to investigate Δspa33/pMyc-spa33, Δspa33/pMyc-yscQ, or Δspa33/pMyc-hrcQB together with Δspa33/pMyc-spa33 for the ability to rescue secretion of IpaB, IpaC, and IpaD into the CR supernatant. Δspa33/pMyc-spa33 rescued defective Δspa33 function, but Δspa33/pMyc-spa3O, Δspa33/pMyc-yscQ, and Δspa33/pMyc-hrcQB did not (Fig. 5B). Thus, although SpaO shares significant amino acid homology with Spa33 (supplemental Fig. S1) and has the capacity to interact with Shigella TTSM-associated proteins except MxiG (12, 21), it was unable to rescue the defective Spa33 function in Shigella.

DISCUSSION

In the present study, we investigated the role of Spa33 in Shigella TTSM formation and protein translocation and found structural and functional evidence to support the concept that Spa33 is an essential component of the C-ring structure involved in TTSM-associated protein translocation. Although it has been proposed that Spa33 (or its homologues) is a component of the TTSM C-ring structure (3, 9, 12, 26), no direct evidence has yet been obtained for bacterial TTSM. Thus, to our knowledge, the present study is the first to provide convincing evidence to support this notion. This conclusion is based on the following findings: (i) the spa33 deletion mutant of Shigella produces a needleless TTSM structure and is unable to secrete any effectors via the TTSM; (ii) Spa33 is located beneath the TTSM, being associated with the two major inner membrane positional TTSM components (MxiG and MxiI); (iii) although Spa33 is incapable of interacting with needle components (MxiH and MxiI), it interacts with TTSM-associated pro-
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teins (MxiN, Spa32, and Spa47) required for needle formation; (iv) Spa33 is capable of interacting with effectors (IpaB, IpaC, IpqB1, and VirA); (v) some residues in the Spa33 C-terminal portion, which are highly conserved among Spa33 homologues, are involved in the interaction with MxiN required for TTSM needle formation; and (vi) Spa33 function in the Shigella TTSM is not interchangeable with those of other Spa33 homologues.

Shigella TTSM morphologically resembles the Salmonella flagella hook-basal body structure (4, 6, 8). The TTSM is composed of an 8.4 × 45-nm external needle and a 26 nm in diameter basal body with two upper rings and two lower rings (4, 8). In addition, the macromolecular structure, which is reminiscent of the C-ring structure of the flagella hook-basal body, has been postulated to be located beneath the TTSM basal body. However, although a C-ring-like structure has been indicated to be associated with the basal body, based on observation of osmotic-shocked Shigella envelopes by TEM (27), there have been no clear demonstrations of a C-ring structure in extensively purified TTSM. Indeed, purified TTSM from S. flexneri, Salmonella typhimurium, or enteropathogenic E. coli consistently showed no C-ring-like macromolecular structure (4, 5, 7, 8, 28, 29), suggesting that the C-ring structure may be lost through the process of TTSM purification, during which such a macromolecular structure might easily be detached from the basal body. Therefore, in this study, we attempted to visualize Spa33 in the vicinity of the bacterial cytoplasmic membrane using immunogold EM or in purified TTSM from Shigella using immunoblotting with anti-Spa33 antibody and confirmed Spa33 to reside in the cytoplasmic TTSM component. In addition, Spa33 was shown to be associated with MxiG and MxiJ in the GST pull-down assay, in which the association with MxiG appeared to be significantly stronger than that with MxiJ. This might be due to the cytoplasmic MxiG domain, which is larger than that of MxiJ. Furthermore, we attempted to directly demonstrate the presence of the macromolecular structure, including Spa33, by stabilizing the bacterial envelope containing TTSM with cross-linking reagents such as dithiobis(succinimidy l) propionate, and the purified TTSM was observed by TEM. We detected macromolecular structures existed beneath the TTSM basal body prepared from Δspa33/pMW-Myc tagging Spa33, which is proficient in TTSM needle formation. However, none were found in the TTSM structure from Δspa33.3 It is noteworthy that the macromolecular structure was stained by immuno-gold, which reacted with Myc-Spa33 (Fig. 2E). Based on the results of a series of experiments, we concluded that Spa33 is a component of the putative C-ring structure and assumed the C-ring structure to be weakly associated with the lower portion of the TTSM basal body via the interactions with MxiG and MxiJ.

There have been no reports on TTSMs of plant pathogens with the C-ring structure, although HrcQb of Pseudomonas, a Spa33 homologue, was recently shown to be associated with some TTSM cytoplasmic components (21). The C-ring of flagella has been indicated to be composed of FlgI, FlgM, and FlhN (6, 30, 31), with FlhN sharing some amino acid similarity with the C-terminal portion of Spa33, as in, for example, Salmonella SpaO, Yersinia YscQ, and Pseudomonas HrcQb (3). Genetic and functional analyses indicated the flagella C-ring to be involved in protein secretion and the regulation of hook length (32), although it remains unclear how the C-ring structure engages in forming the needle structure or in the transit of protein translocated via the TTSM. The flagella C-ring has been proposed to act as “a measuring cup,” providing binding sites on the inner wall surface to hold flagella hook subunits, thus enabling the hook-basal body to form a hook of uniform length (32). However, another mechanism for determining the length of the TTSM needle has been proposed in Yersinia TTSM (33, 34). Indeed, in both flagella and the TTSM there is an additional protein acting as a molecular ruler, e.g. flagella FliK, Shigella Spa32, or Yersinia YscQ (19, 33, 35). Since we did not observe Spa33 to directly interact with the needle components MxiH or MxiL, interacting instead with Spa32, Spa47, MxiK and MxiN, the latter must be involved in formation of the TTSM needle. Jouihri et al. (14) previously reported that MxiN and MxiK are capable of interacting with Spa47 and are required for transit of the needle components MxiH and MxiL through the TTSM. Although the exact roles of MxiN and MxiK in transit of the needle components are not known, MxiN and MxiK together with Spa33 might be components of the C-ring.

The C-terminal portion of Spa33 shares significant homology with FlhN, SpaO, YscQ, and HrcQb, in which some of the amino acid residues of Spa33, such as residues 226, 242, 266, 267, 269, 273, 276, 283, and 288, are highly conserved among Spa33 homologues (see Fig. 4A). By creating the single amino acid substituted mutant of each of the conserved residues of Spa33, we investigated the effects of these substitutions on TTSM formation and its secretion activity. Three residues, 242, 273, and 286, were found to be the most critical residues, since each substitution resulted in the formation of a needle-less TTSM that was unable to secrete any of the Ipa proteins into the medium. Of note, each single amino acid substituted Spa33 variant was still able to interact with Spa32 or Spa47, but not MxiN, in the GST pull-down assay. Since knocking out of the mxiN gene in Shigella produced a needle-less TTSM, which was unable to secrete Ipa proteins (14),3 it is likely that the Spa33-MxiN interaction mediated by the C-terminal portion of Spa33 is important for transit of the needle component through the TTSM. Since Spa33 function was not interchangeable with those of other Spa33 homologues in terms of Shigella TTSM activity, Spa33 apparently has the capacity to interact with multiple proteins associated with TTSM and translocating effector proteins. We thus assume that Sap33 is an essential component of the C-ring and serves as the platform mediating the transit of proteins to be transported through the TTSM basal body. Our analysis suggests a model for the role of Spa33 in the morphological pathway of TTSM formation (supplemental Fig. S2). Analogously with the formation of the basal body of the Salmonella flagellum or TTSM (6, 18, 36), several Shigella Mxi proteins are anchored in the inner and outer membranes and build up the basal body (4, 8, 28). The C-ring compartment, which is possibly composed of Spa33 (this study), must be necessary to form the functional secretion apparatus, since the spa33 mutant is incapable of forming the needle structure or mediating the secretion of effector proteins. C-ring formation could take place after formation of the basal body, since in the absence of Spa33 the bacterium is still capable of forming a defective basal body structure within the envelope (this study). The functional basal body may subsequently mediate translocation of MxiL (a putative needle component embedded in the basal body) and MxiH (the major surface-exposed needle component) to extend needle structure with the aid of Spa47 ATPase (4, 8, 37), in which Spa32 may act as a molecular ruler, similar to Yersinia YscP, to determine the length of the needle (19, 33). The formation of functional TTSM may eventually be accomplished by releasing Spa32 from the needle into the space surrounding bacteria (19), which may be followed by translocation of IpaB, IpaC, and IpaD proteins via the TTSM to act as the molecular cap at the tip of the needle (9). Although the precise mechanism underlying the recognition of its own translocating proteins remains to be elucidated, the specificity of Spa33 for its own TTSM and the ability to interact with multiple proteins are key issues in the protein sorting system operated by TTSM.
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