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Ultrastructural analysis of IpaD at the tip of the nascent MxiH type III secretion apparatus of *Shigella flexneri*

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Abstract

Shigella flexneri is a Gram-negative enteric pathogen that is the predominant cause of bacillary dysentery. *Shigella* uses a type III secretion system (T3SS) to deliver effector proteins that alter normal target cell functions to promote pathogen invasion. The type III secretion apparatus (T3SA) consists of a basal body, an extracellular needle, and a tip complex that is responsible for delivering effectors into the host cell cytoplasm. Invasion plasmid antigen D (IpaD) is the first protein to localize to the T3SA needle tip, where it prevents premature effector secretion and serves as an environmental sensor for triggering recruitment of the translocator protein IpaB to the needle tip. Thus, IpaD would be expected to form a stable structure whose overall architecture supports its functions. It is not immediately obvious from the published IpaD crystal structure (pdb 2j0o) how a multimer of IpaD would be incorporated at the tip of the first static T3SA intermediate, nor what its functional role would be in building a mature T3SA. Here we produce three-dimensional reconstructions from transmission electron microscopy images of IpaD localized at the *Shigella* T3SA needle tip for comparison to needle tips from a *Shigella ipaD*-null mutant. The results demonstrate that IpaD resides as a homopentamer at the needle tip of the T3SA. Furthermore, comparison to tips assembled from the distal domain IpaD^{Δ192-267} mutation shows that IpaD adopts an elongated conformation that facilitates its ability to control type III secretion and stepwise assembly of the T3SA needle tip complex.

Keywords

tip protein; type III secretion system; *Shigella*; IpaD; type III secretion apparatus

INTRODUCTION

Shigella is a Gram-negative bacterial pathogen and the causative agent of shigellosis, a severe bacillary dysentery characterized by bloody, mucoid diarrhea ¹. It is estimated that there are more than 160 million cases of shigellosis annually throughout the world with the

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major at-risk group being children under the age of five in the developing world. In addition to high morbidity and mortality in children, shigellosis is often associated with long-term impairment of cognitive and physical development². The lack of an efficacious vaccine that prevents *Shigella* infection and is safe for children makes identification of the major contributors to pathogenesis imperative.

Like many other important Gram-negative pathogens, *S. flexneri* uses a type III secretion system (T3SS) to deliver protein effectors into targeted human cells for the benefit of the bacterium³. For *Shigella*, this benefit is pathogen entry into the epithelial cells of the human colon, which provides a site for bacterial multiplication. The type III secretion apparatus (T3SA), which refers to the structural components of the T3SS, resembles a needle and syringe that provides an energy-driven unidirectional conduit from the bacterial cytoplasm to the target cell membrane and cytoplasm. The T3SA needle, with an external diameter of ~7.0 nm and an inner diameter of ~2.5 nm, is formed by the addition of MxiH monomers at the distal end until it extends just beyond the lipopolysaccharide (LPS) layer⁴. The tip of the nascent T3SA needle is capped by a needle tip complex that is initially comprised of a single protein—invasion plasmid antigen D (IpaD)⁵. Following recognition of small molecules such as bile salts, IpaD undergoes a conformational change that promotes recruitment of the hydrophobic IpaB translocator protein to a location distal to IpaD. IpaB subsequently serves to detect the cholesterol-rich environment of the host membrane to promote the localization of the second hydrophobic translocator protein, IpaC, to the T3SA needle tip⁸. This event completes the formation of the translocon and the T3SA, which is now fully open and competent to transport effector proteins from the *Shigella* cytoplasm into the host cytoplasm⁸.

T3SA tip proteins can be minimally divided into three families: a) the IpaD family characterized by the tip proteins from *Shigella*, *Salmonella* and some *Burkholderia* species; b) the LcrV family characterized by the T3SA needle tip proteins from *Yersinia* species and *Pseudomonas aeruginosa*; and c) the EspA family characterized by tip proteins from enteropathogenic *Escherichia coli* and related pathogens⁹. All confirmed T3SA tip proteins are characterized as having an overall dumbbell shape with the handle formed by an antiparallel coiled-coil that provides rigidity and is presumably necessary for needle tip docking. In contrast, each family has unique globular domains located at the ends of the dumbbell handle that are likely responsible for pathogen-specific functions⁹. The large globular domains of LcrV have allowed the structure of the ring atop the needle to be clearly visualized¹², however, similar structural information for IpaD has not been so easy to obtain.

IpaD is a 36-kDa globular protein with a stabilizing central coiled-coil, an N-terminal domain that has been proposed to have a self-chaperoning function and a distal domain that has been linked to secretion control and maintenance of IpaB at the T3SA needle tip¹⁰. IpaD has been proposed by our group to reside as a multimeric ring structure at the tip of the MxiH needle¹⁰. In contrast to LcrV, the globular domains of IpaD are elongated, which has proven to be an obstacle in visualizing IpaD at the *Shigella* T3SA needle tip. In this report, we present the first evidence for an IpaD ring structure having five-fold symmetry at the tip of the MxiH needle. Formation of this pentameric complex requires that the IpaD distal domain is extended upward relative to what has been observed in the IpaD crystal structure. This is in contrast to the position of the distal domain adjacent to the central coiled-coil in the static crystal structure of monomeric IpaD¹⁰. Furthermore, we present structures of the MxiH needle end from an *ipaD* null strain and an IpaD distal domain deletion mutant for comparative purposes. Biochemical analysis and imaging of this IpaD distal domain deletion mutant support our hypothesis that this distal domain is essential to prevent premature T3SA secretion.

RESULTS

The IpaD distal domain is required to maintain a closed channel for the nascent tip complex

Deletion of the distal domain of IpaD, IpaD^{Δ192-267}, gives rise to a strain that cannot control secretion or invade HeLa cells but still maintains IpaD at the T3SA needle tip (data not shown). To examine the role of the distal domain in more detail, we took an approach that removed portions, rather than the entire, distal domain following nascent tip complex assembly. Paired Tobacco Etch Virus (TEV) cleavage sites were inserted into looped regions within the IpaD distal domain to allow removal of sections of this domain following localization to the *Shigella* T3SA needle tip (Fig. 1b and Supplemental Fig. 1). Mutants containing sites three and four, IpaD^{TEV3/4}, allowed removal of helix 5 and β -sheets C and D at the end of the distal domain while sites five and six, IpaD^{TEV5/6}, left only the triple β -sheet structure atop the coiled-coil¹⁰. To ensure that the proteins could be cleaved by TEV as predicted, recombinant proteins were prepared from *E. coli*. After using circular dichroism to determine that the secondary structure was not compromised for these mutants (data not shown), IpaD^{TEV3/4} and IpaD^{TEV5/6} were treated with TEV. Both proteins exhibited significant cleavage, while wild type IpaD did not (Fig. 1a) indicating that the engineered TEV cleavage sites were available for cleavage.

The corresponding pWPsf4 plasmids containing the *ipaD*^{TEV3/4} and *ipaD*^{TEV5/6} mutant genes were then used to complement the *S. flexneri ipaD* null strain, SF622. Both strains exhibited surface localization of the mutant IpaD, which remained localized following TEV treatment, as assessed by immunofluorescence microscopy (Fig. 1c). When other phenotypic characteristics were examined, the strain expressing IpaD^{TEV5/6} did not invade HeLa cells (8±5% of control) in a standard gentamicin protection assay. Additionally, assessment of secretion indicated a loss of control for secretion of the translocators, IpaB and IpaC. Presumably, the addition of the TEV sites, while not affecting tip localization, did negatively affect secretion control even before TEV-mediated removal of the region, rendering this strain unacceptable for further studies examining secretion control. In contrast, the strain expressing IpaD^{TEV3/4} complemented invasion of HeLa cells to nearly wild type levels (88% ±10 of control) and maintained secretion control before TEV cleavage, indicating that the insertion of the TEV cleavage sites did not negatively influence the physiological role of IpaD. Therefore, SF622 cells expressing IpaD^{TEV3/4} were used to assess secretion control by the distal domain. Control of secretion by SF622 expressing IpaD^{TEV3/4} was assessed by first growing the bacteria to mid-log phase and resuspending them in PBS. The resuspended bacteria were then exposed to different amounts of TEV protease and incubated for 30 min, followed by trichloroacetic acid (TCA) precipitation of the secreted proteins and quantification by western blot analysis (Fig. 2). While the amounts of IpaB and IpaC secreted are essentially the same for *Shigella* expressing IpaD^{TEV3/4} and the wildtype IpaD, the secretion of IpaB and IpaC increases more than two-fold after 15 μ g of TEV is added to the strain expressing IpaD^{TEV3/4} ($\alpha=0.1$). Unlike the *Shigella* strain expressing IpaD^{Δ192-267} whose overall structural integrity was unknown, the cleavage of the IpaD TEV mutants by TEV in real time indicated that the distal domain plays an important role in type III secretion control.

IpaD forms a pentameric ring

We have previously shown that IpaD localizes to the T3SA needle tip⁵. Morphological differences were observed in transmission electron micrographs of wild-type nascent needle tips and *ipaD* null T3SA needle ends (Fig. 3a and b, bottom rows). To determine the structure of the IpaD T3SA tip complex and to elucidate the mechanism by which IpaD controls secretion, we have resolved the tip structure of the nascent T3SA needle tip

complex from needles isolated from wild-type *Shigella* for comparison to needles from an *ipaD* null strain. To compute 3D reconstructions, 835 and 940 needle ends were selected from micrographs of wild-type and null samples, respectively. These particles (e.g. Fig. 3 bottom rows) were then aligned and classified. After computing an initial 3D reconstruction, the process was repeated iteratively and final 3D reconstructions were then calculated from the final class averages (e.g. Fig. 3 top rows).

The helical symmetry of the *S. flexneri* T3SA needle is 5.6 subunits per turn of the helix¹³. Scanning transmission electron microscopy measurements of LcrV, the *Yersinia pestis* IpaD homolog, shows a molecular mass that would correlate with the tip complex having 5 copies of LcrV with 5-fold symmetry¹⁴. Conversely, Johnson *et al.* suggested that the *Shigella* needle tip complex has four IpaD monomers and one IpaB monomer¹⁰. We reasoned that since one IpaB monomer is approximately twice the mass of one IpaD monomer, pseudo-6-fold symmetry may account for the mass of the proposed tip complex structure. Therefore, needle tips composed of IpaD were tested for no symmetry and then for 4-fold, 5-fold and 6-fold symmetry. Using Fourier Shell Correlation (FSC) curves (Supplementary Fig. 2) we determined that the wild-type needle tip complex possesses five IpaD monomers in 5-fold symmetry whereas the *ipaD* null mutant has no centrosymmetric symmetry.

After ten iterations of refinement, 3D reconstructions were computed and scaled to match the known diameter of the needle⁴ (scaling <10%) to accommodate magnification differences due to lack of stage eucentricity and/or shrinkage due to stain. The threshold for each reconstruction corresponds to the expected protein mass for the assembly, assuming a protein density of 1.23Å³/Da. The final 3D reconstructions are shown in figure 4, for the needles from wild-type *Shigella* and an *ipaD* null strain, at resolutions of 20Å and 24Å, respectively. The wild type tip complex has an inner diameter at its widest point of 40Å and 22Å at its narrowest point. It possesses a collar at the IpaD-needle interface that is absent on the *ipaD* null needle (Fig. 4 black arrow). The density of the wild-type tip complex extends 95Å axially above this collar, with five rods extending upward and wrapping inward to form a “scepter”-like structure. The largest outer diameter of the scepter is 78Å. None of these densities are observed in the *ipaD* null mutant, but rather the needle appears to collapse on itself to form a “pencil”-like structure (Fig. 4b).

The distal domain of IpaD is extended upward and inward

To correlate the 5-fold symmetry of the needle tip complex with the published crystal structure of IpaD¹⁰, five copies of IpaD were modeled into the 3D reconstruction. No satisfactory fit was possible using the original crystal structure. Therefore, an automated segmentation algorithm from UCSF Chimera¹⁵ was used to identify domains within the density map. This segmented map was then used to model the crystal structure into the map by the sequential fitting of individual IpaD domains. In contrast to the monomeric crystal structure, the resulting pentameric model displays each IpaD as further elongated with the distal domain directed upward to form an inward wrapping at the top of the density. A large loop region proximal to the distal domain allows this approximately 165° rotation, as illustrated in Fig. 5c. The central coiled-coil of IpaD fits into the map angled at 20° from the needle axis, allowing intercalation of the proximal needle protein MxiH around the collar region. As in the solved crystal structure, the N-terminal domain of IpaD remains proximal to the central coiled coil region (Fig. 5a). To fit into the EM density map, this domain is rotated from its position in the crystal structure approximately 165° about the long axis of the molecule (Fig. 5c). Due to its short length and flexible tether, we expect the 2' helix (residues 108–117) to accommodate this rotation, however due to the limited resolution of our data, specific localization of this domain was not attempted. With its proximal localization, the N-terminal domain takes the place of a MxiH needle protein, thereby anchoring IpaD onto the needle tip, as we have described previously¹⁶. Furthermore, the N-

terminal domain remains surface exposed (Fig. 5a), in agreement with previous experiments probing surface accessibility of the N-terminus via immunofluorescence microscopy using monoclonal antibodies against residues 30–45. Thus, the placement of this region as proposed here is consistent with all of the known physiological data.

Unfortunately, the resolution of our reconstruction of the IpaD needle tip was not sufficient to unambiguously orient IpaD subunits rotationally about the long axis of the molecule and unequivocally demonstrate the position of the distal domain. This problem was resolved using IpaD Δ 192-267, a deletion of the distal domain¹⁰, to help identify the position of the IpaD distal domain within the nascent needle tip complex. Although the SF622 strain expressing IpaD Δ 192-267 is unable to invade cultured cells or lyse red blood cells and exhibits a moderate hypersecretion phenotype (data not shown), IpaD is surface localized for this strain during the first static state¹⁰. Thus, the distal domain probably has a role in controlling the temporal assembly of the T3SA and secretion. As was done for the wild-type and *ipaD* null mutant, 604 needle tips were selected from electron micrographs and particles were aligned and classified (Fig. 6a). Like wild type, the 22Å resolution IpaD Δ 192-267 structure (Fig. 6b) possesses 5-fold symmetry and maintains the collar at the IpaD-needle interface. Unlike the wild-type structure, the upward extending rods are shortened, with a collar to tip length of 58Å, and the inward wrapping is no longer present. In this mutant structure, the largest outer diameter has decreased to 70Å as compared to 78Å for wild-type complexes and the inner diameter of the mutant at the tip is 30Å. Not surprisingly, the N-terminal and central coiled-coil domains fit in the mutant reconstruction much as they did in the wild-type structure when the distal domain was present (Fig. 5b). In Figure 5, numbers 1–6 are 15Å horizontal slices taken at different planes as indicated by the black arrows. Examination of the sections of the two models not only shows that there is sufficient space in the density for the coils of the crystal structure without blocking the central channel, but also that the distal domain wraps inward to close the T3SA channel.

To better understand the structure of T3SA before and after the addition of the IpaD tip protein, we compared the null, wild-type, and mutant IpaD needle end regions (Fig. 7). As seen in Figures 4 and 7, in the absence of IpaD the needle has a constricted end (red). In the structure that includes IpaD (blue mesh) and IpaD Δ 192-267 (green transparent) this region has an open central channel at the collar, 25Å in diameter. This collar region likely includes both MxiH and IpaD as indicated by sufficient density in this region of the wild-type IpaD needle tip to accommodate both proteins. The closed tip of *ipaD* null, as compared to the open central channels of the IpaD-containing tips suggests that there is a conformational change of the needle upon addition of IpaD. This transition to an open channel is likely a consequence of the interaction between MxiH and IpaD at the collar. When the collar regions of wild-type IpaD and IpaD Δ 192-267 are aligned, wild type IpaD protrudes distally 37Å beyond the mutant IpaD. Reconstruction of IpaD Δ 192-267 needle tip complexes not only demonstrates the location of the distal domain but also implicates the distal domain in the control of type III secretion. These results further support our hypothesis that the distal domain of IpaD is elongated and forms the inward wrapping of the wild type IpaD reconstruction, preventing premature secretion by the nascent T3SA.

DISCUSSION

The T3SA is a complex nanomachine for which many aspects of structure and function are still enigmatic. The *Shigella* needle tip complex, for example, has been difficult to dissect and the translocator proteins which are ultimately recruited to the needle tip are hydrophobic and only stable when complexed with a chaperone. The homologous LcrV tip complex from *Yersinia* was first described in Mueller *et al.* and was visualized as a hat-like structure¹², but no similar structure has been seen at the *Shigella* needle tip. Here we present the first 3D

reconstruction of the nascent *Shigella* T3SA IpaD tip complex. IpaD is observed as an elongated pentamer which causes the needle tip to resemble a scepter. In contrast, an *ipaD*-null mutant reconstruction resembles a pencil tip, in which the end is collapsed to a point. Though not observed here, the naked needle tip probably possess a great deal of flexibility since *ipaD*-null strains are uncontrolled with regard to secretion⁵.

In this study we show that IpaD at the tip of the T3SA forms a pentamer with five rods extending upward and ultimately wrapping inward. The rods extend upward 95Å from a collar region that marks the IpaD-MxiH collar region. It has been proposed that it is possible to fit four IpaD monomers along with one IpaB monomer at the tip, which was suggested to give rise to an open secretion channel¹⁰. Since these studies began, however, we have determined the crystal structure for the N-terminal region of IpaB, which contains an 114Å long coiled-coil¹⁷. Knowing that IpaB is approximately twice the mass of IpaD and would extend further upward from the needle tip than IpaD does, we expect that a monomer of IpaB is added to the needle tip after addition of deoxycholate, and does not displace IpaD monomers that we observe at the tip of the nascent T3SA needle. In support of this, IpaB co-localizes at the needle tip with IpaD, but IpaB is present only after addition of deoxycholate⁷.

The reconstruction of the wild type IpaD tip shows an inner needle diameter of 25Å that opens up to 40Å before closing back down at the distal tip to 22Å. This large open inner diameter in the tip may allow small portions of protein to partially fold as they prepare to exit the channel. We speculate that this mechanism, coupled with the constricted 22Å tip may be effective in stopping premature secretion of translocator and effector proteins. It is also possible that no partial folding takes place and the closed tip is still sufficient to block translocators and effectors exiting the needle.

The T3SS is known to share characteristics with the flagellar export system. Like the T3SS, the flagellar system is constructed at its distal end. Flagellar assembly uses a cap protein to facilitate proper placement of the filament monomers. Our work is the first to suggest that the T3SS may not use a cap protein for needle assembly. The needle appears to form a “closed”, pointed end before the addition of IpaD. This pencil-like end is likely to possess innate flexibility, however, it may slow the exported needle monomers to allow sufficient time to assume proper placement at the growing end of the needle. With the addition of IpaD, the needle end is now held open, and our data indicate that it is the IpaD distal domain that blocks (and controls) premature secretion.

Previous studies have used the crystal structure to depict IpaD modeled as a pentamer, but the placement of the N-terminal and distal domains made the pore impassable without major structural rearrangements¹⁰. In this study, when the density map was segmented, the only satisfactory fit found was one in which the IpaD distal domain is elongated and positioned above the central coiled-coil. The central coiled-coil is situated at a 20° angle from the needle axis in our model and the N-terminal domain remains proximal to the coiled-coil. We also computed a reconstruction of a distal domain mutant, IpaD^{Δ192-267} to better determine the orientation of IpaD and its distal domain within the tip structure. Using these data, the N-terminal domain is modeled as surface exposed, which is consistent with previously published results. When overlaid with the collars aligned, the wild-type and IpaD^{Δ192-267} reconstructions show a difference in length of approximately 37Å from collar to tip. Also, there is no inward wrapping seen for IpaD^{Δ192-267}, which suggests that we have modeled this domain correctly. Taken together these results indicate that the distal domain may be used by the T3SS to block premature secretion. The fit of IpaD in the reconstructions prompted us to use biochemical means to examine in detail which specific regions of the distal domain are necessary for secretion control.

The proposal that the IpaD distal domain controls secretion was confirmed by phenotypic evaluation of IpaD mutants in which TEV cleavage sites were introduced in the distal domain. These studies further implicate the distal domain in prevention of premature secretion of effectors. When a portion of the distal domain is removed, IpaD^{TEV3/4}, the mutant continues to be localized at the needle tip but it loses its ability to control secretion. Thus, from its position beyond the coiled-coil, the distal domain appears to be responsible for maintaining secretion control as part of the first static intermediate of the T3SA needle tip complex, perhaps by forming a constricted channel conformation.

The findings presented here are important in elucidating the mechanisms by which IpaD contributes to secretion control and early steps of T3SS activation and induction. Many Gram-negative bacteria employ the T3SS as an essential component of their virulence arsenals. Thus, gaining insight into the structure and function of the T3SA tip complex would provide a significant advance in our understanding of virulence in a range of pathogens. Our reconstruction shows that immature needles (comprised of just MxiH) are closed, which may explain why it has not been possible to identify a cap that contributes to needle assembly, as has been observed for flagellar filament assembly. Furthermore, since IpaD has been shown to be a protective antigen²⁰, the elongated conformation proposed here for IpaD, with its exposed N-terminal domain may allow for the targeted development of vaccines against shigellosis.

MATERIALS AND METHODS

Materials

S. flexneri ipaD null mutant (SF622) was a gift from P.J. Sansonetti (Institut Pasteur, Paris, France)²¹. *mxiH* null mutant strain (SH116) was a gift from A. Allaoui (Brussels, Belgium)²². The plasmids pBAD24 and pBAD33 were a gift from G. Munson (University of Miami). TEV protease was a gift from B. V. Geisbrecht (University of Missouri, Kansas City). Oligonucleotides were purchased from IDT (Coralville, IA). Competent *E. coli* cells, ligation mix and pET15b were from Novagen (Madison, WI). Restriction enzymes were from New England Biolabs (Tozer, MA).

Preparation of plasmids for protein expression in *S. flexneri* and *E. coli*

pWPsf4D containing the *ipaD* gene has been described²³. pWPsf4D^{Δ192-267} was made by inverse PCR using pWPsf4D as template and primers composed of GAGAGA, a XhoI restriction site and 18 nucleotides flanking the region to be deleted. The PCR product was digested with XhoI, intramolecularly ligated, and transformed into *E. coli* NovaBlue.

To express IpaD with dual TEV cleavage sites, the native SfoI site in pWPsf4D was mutated to a SmaI site resulting in pWPsf4.2D. Inverse PCR was used to introduce a SfoI restriction site in the first of the two locations to contain TEV cleavage site insertions in the gene. The PCR product was digested with SfoI, intramolecularly ligated, and transformed into *E. coli* NovaBlue. The resulting plasmid was digested and the annealed oligonucleotides containing the TEV cleavage sequence (GAAAATCTTTATTTCCAAGGA) ligated in, simultaneously destroying the SfoI site. The ligated product was used to transform *E. coli* NovaBlue. The second TEV site was inserted in the same manner. Two dual TEV site pWPsf4D plasmids were constructed. The plasmid pWPsf4D^{TEV3/4} allowed removal of residues 206 to 250 and plasmid pWPsf4D^{TEV5/6} allowed removal of residues 191 to 261. The mutant *ipaD* genes were subcloned into pET15b and the resulting plasmid transformed into *E. coli* Tuner(DE3) for overexpression and purification of the IpaD-TEV proteins. The sites at which TEV sequences were inserted are indicated in Supplemental Fig. 1.

Construction of *mxiH*/pWPsf4 (pRK*mxiH*) has been described ²⁴. The *mxiH* gene was subcloned into pBAD24 ²⁵. To create a MxiH expressing plasmid that was compatible with pWPsf4, a Shine-Delgarno and NdeI site were engineered into pBAD33²⁵ between the EcoRI and SacI sites giving rise to pBAD33ND₂. *mxiH* was then inserted into the NdeI and HindIII sites.

The plasmid *mxiH*/pBAD24 was electroporated into *S. flexneri* SH116 (a *mxiH* null) to yield wild type *ipaD* atop long needles. The plasmid *mxiH*/pBAD24 was electroporated into *S. flexneri* SF622 (an *ipaD* null) to yield long needles without IpaD. The pWPsf4DTEV plasmids were electroporated into SF622. Ampicillin selection ensured the presence of the plasmid while kanamycin resistance and/or Congo red binding indicates presence of the *Shigella* virulence plasmid. The *mxiH*/pBAD33ND₂ and pWPsf4DΔ192-267 plasmids were electroporated into *S. flexneri* SF622 cells to yield IpaD^{Δ192-267} atop long needles. In addition to ampicillin, chloramphenicol was used to select for the pBAD33ND₂ plasmid. Complementation was assessed as needed using the gentamicin protection assay as previously described ²⁶.

Sample preparation, Imaging, and Data Digitization for TEM

When *mxiH* is subcloned behind the inducible *arabinose* promoter in pBAD24 or pBAD33ND₂ and protein expression is induced, long needles greater than 300 nm are produced. After growth to late log phase, bacteria were harvested and resuspended in PBS. A portion of the sample was fixed in 2% paraformaldehyde/2% glutaraldehyde prepared in 0.3M sodium phosphate for 30 min and another portion was left unfixed; no difference was seen in initial 3D EM reconstructions of fixed and unfixed samples analyzed separately. Bacteria were washed once and resuspended in PBS. The suspension was forced through a 21G needle to mechanically shear the needles from the bacteria. Samples were centrifuged twice at 20,000xg, collecting the needle-rich supernatant each time. Needles were then concentrated by ultrafiltration, and further purified using a 10%–30% sucrose step gradient. The 30% sucrose fraction was collected and dialyzed against TE pH 7.6 or HEPES pH 7.6.

Grids for electron microscopy (EM) were prepared by adsorption of 4 μl of sample onto glow-discharged carbon coated grids. The grids were washed with TE pH 7.6 and negatively stained with 1% uranyl acetate. Images were taken under low electron dose conditions at a magnification of 60,000x on a Philips CM-12, recorded on Kodak SO-163 film, developed in full-strength D19 (Kodak), and digitized with a Nikon Super CoolScan 9000ED at 4,000 dpi for a final pixel size of 4.23 Å.

Image processing

EMAN (Electron Microscope Analysis Software) was used for two-dimensional reconstruction and EMAN2 was used for three-dimensional reconstruction using single particle analysis. First, particles were picked with the tip of the needle centered in an 80x80 pixel box. Those boxes were then stacked, aligned, and averaged in two dimensions. The resulting 2D projection was low pass filtered and used to produce a cylindrically symmetric initial reference model for 3D reconstruction. 3D reconstructions of each sample, including wild type IpaD, *ipaD* null, and IpaD^{Δ192-267}, were computed using iterative alignment, classification, and reconstruction in EMAN2. A cutoff of 0.5 was employed in EMAN2 to include only the best 50% of the particles in the final 3D reconstructions.

Cleavage with TEV protease

ipaD^{TEV3/4}/pET15b and *ipaD*^{TEV5/6}/pET15b in Tuner(DE3) were grown and purified as previously published ²⁹. IpaD^{TEV5/6} was dialyzed into PBS directly following immobilized metal ion adsorption chromatography (IMAC) purification. IpaD^{TEV3/4} was extracted from

inclusion bodies by denaturation in 6M urea, purified via IMAC, and refolded by step dialysis into PBS. Purity and proper secondary structure were confirmed by SDS-PAGE and far-UV circular dichroism spectroscopy (Jasco J815 spectropolarimeter), respectively, as previously described ²⁰.

IpaD^{TEV3/4} and IpaD^{TEV5/6} (0.85mg/ml) in PBS containing 5mM β -mercaptoethanol were each cleaved with 5 μ g of TEV overnight at room temperature. Cleavage was analyzed by SDS-PAGE. *S. flexneri* expressing IpaD containing the TEV sites were grown to mid-log phase and resuspended in PBS containing 1 mM β -mercaptoethanol. The suspension was divided into three aliquots and the indicated amount of TEV added. After 30 min at 32°C to allow TEV cleavage, the bacteria were moved to 37°C for an additional 30 min to further promote uninduced secretion. The culture supernatant was then collected and the proteins of the supernatant were precipitated with TCA and analyzed by SDS-PAGE and western blot. IpaB, IpaC and IpaD were detected using mouse monoclonal or rabbit polyclonal antibodies followed by Alexa Fluor 680 goat anti-mouse or anti-rabbit IgG (LI-COR, Lincoln, NE). Western blot images were obtained using an Odyssey Infrared Imager (LI-COR, Lincoln, NE) and analyzed with Odyssey Infrared Imaging System Application Software v3.0.21 (LI-COR, Lincoln, NE).

Fluorescence Microscopy

Immunofluorescence microscopy was performed as described ⁵. Briefly, bacteria were chemically fixed and probed with rabbit anti-IpaD polyclonal antibody, followed by goat anti-rabbit Alexa Fluor 488 secondary antibody. Images were captured with an Olympus IX81 inverted microscope equipped with a Hamamatsu ORCA-R² CCD camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T3SS	type III secretion system
T3SA	type III secretion apparatus
Ipa	invasion plasmid antigen
EMAN	Electron Microscopy ANalysis
Lcr	low calcium response
Esp	<i>Escherichia coli</i> secretion protein
Mxi	major exporter of Ipa
TEV	tobacco etch virus
FSC	Fourier Shell Correlation

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- First 3D reconstructions from TEM images of IpaD at the T3SA needle tip
- IpaD forms a homo-pentameric ring structure at the tip of the T3SA needle
- IpaD has five fold symmetry at the T3SA needle tip
- IpaD distal domain is extended upward relative to the crystal structure (pdb 2J0o)
- The IpaD distal domain is necessary to prevent T3SA premature secretion.

Figure 1. IpaD cleavage and localization to the T3SA needle tip

Coomassie stained SDS-PAGE of wild-type IpaD, IpaD^{TEV3/4}, and IpaD^{TEV5/6}, before and after TEV exposure (a). The IpaD crystal structure is shown with the distal domain highlighted in cyan and TEV site insertion pairs shown by colored spheres. TEV sites 3 and 4 are shown in blue and sites 5 and 6 are shown in yellow (b). Immunofluorescence microscopy of TEV mutants is shown in (c). The top two rows are after exposure to TEV, while the bottom two rows were similarly treated but without TEV. The top row in each set are the phase-contrast images indicating the presence of bacteria and the bottom row in each set are immunofluorescence images indicating whether IpaD remains on the bacterial surface after treatment (c).

Figure 2. IpaD distal domain is responsible for T3SS translocator secretion control

Western blot analysis of wild-type and IpaD^{TEV3/4} complemented *S. flexneri* secreted translocator proteins following exposure to increasing amounts of TEV protease. The experiment was repeated in triplicate and the bands were quantified by densitometry. Exposure of the *ipaD*-complemented SF622 *ipaD*-null strain to 15 μg of TEV resulted in little change of translocators' secretion profiles (80±7% and 74±17% of wild-type for IpaB and IpaC, respectively). Exposure of the *ipaD*^{TEV3/4} complemented SF622 strain exposed to 15 μg of TEV protease resulted in loss of secretion control and IpaB and IpaC secretion levels measured at 243±18% and 296±43%, respectively, of those not exposed to TEV.

Figure 3. Single particle analysis of wild-type IpaD and *ipaD*-null T3SA

Representative structural classes (top row) and images of single particles (bottom row) comprising wild-type IpaD located on the needle tip (a) and *ipaD*-null needle ends (b). The top row of each panel shows representative class averages while the bottom row of each panel shows a representative single particle from the class image located directly above. The scale bar is 100Å.

Figure 4. Three-dimensional reconstructions of wild-type IpaD and *ipaD*-null T3SA
 Density maps of wild type IpaD (a), and *ipaD* null (b) needle tip reconstruction. The 20Å resolution wild type needle tip has an inner diameter of 40Å at its widest point and 22Å at its narrowest point. The black arrow denotes location of the collar of the wild type IpaD needle tip. Above this collar, the tip extends 95Å, where five rods are seen extending upward and wrapping inward to form a scepter-like structure. None of these attributes are seen in the 24Å resolution *ipaD*-null reconstruction. The tip of the density seems to collapse inward forming a pencil-like structure for the ends of needles from the *ipaD*-null. Wild type IpaD (c) and *ipaD* null (d) needle tip reconstructions are tilted 90° in the positive z plane to show the constricted opening of wild type and the pencil tip of *ipaD* null needle tips. The scale bar is 50Å.

Figure 5. Modeling the IpaD crystal structure into EM density maps

In order to obtain a proper fit for the crystal structure into the wild type IpaD reconstruction, the crystal structure had to be elongated by shifting the distal domain upward (c, pink to red). The central coiled-coil (green) fit in at a 20° tilt from the needle axis. The N-terminal domain remains proximal to the central coiled-coil (a and c, light blue to blue). In order to see the fit, 15 Å thick slices are shown at the locations indicated by numbers and arrows. The crystal structure of IpaD, minus residues 192-267, was fit into the IpaD Δ 192-267 needle tip reconstruction (b). Again the numbers and arrows denote 15 Å slices that were taken to better show the crystal fit.

Figure 6. Single particle analysis of IpaD Δ 192-267

Representative structural classes (top row) and representative images of single particles (bottom row) of IpaD Δ 192-267 located on the needle tip (a). Density map of the IpaD Δ 192-267 needle tip reconstruction at 22Å resolution (b). The black arrow denotes location of the collar region from which five rods extend 58Å upward. Unlike the wild type reconstruction, there is no inward wrapping at the top of this structure as seen when the density map is tilted 90° in the positive z direction. The scale bar is 50Å.

Figure 7. Comparison of needle tip and needle end structures

Reconstructions of wild type IpaD (blue mesh), IpaD $\Delta 192-267$ (green transparent) (a), IpaD-null (magenta solid) and wild type IpaD (blue mesh) (b), and wild type IpaD (blue mesh), IpaD-null (magenta solid), and IpaD $\Delta 192-267$ (green transparent) (c) needle tips were overlayed. The wild type IpaD and the IpaD $\Delta 192-267$ needle tips were aligned based on collar location while *ipaD*-null was aligned at the collar, where the structure is thought to switch from helical MxiH to pentameric IpaD. The scale bar is 50Å.