The cytoplasmic domain of MxiG interacts with MxiK and directs assembly of the sorting platform in the *Shigella* type III secretion system

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Shoichi Tachiyama¹, Yunjie Chang‡, Meenakumari Muthuramalingam, Bo Hu**, Michael L. Barta†‡, Wendy L. Picking**, Jun Liu§, and William D. Picking**†‡³

From the ¹Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045, the ²Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut 06516, the ³Microbial Sciences Institute, Yale University, West Haven, Connecticut 06516, the ⁴Higuchi Biosciences Center, University of Kansas, Lawrence, Kansas 66047, the ⁵Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Texas 77030, and the ⁶Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66047

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Many Gram-negative bacteria use type III secretion systems (T3SSs) to inject virulence effector proteins into eukaryotic cells. The T3SS apparatus (T3SA) is structurally conserved among diverse bacterial pathogens and consists of a cytoplasmic sorting platform, an envelope-spanning basal body, and an extracellular needle with tip complex. The sorting platform is essential for effector recognition and powering secretion. Studies using bacterial “minicells” have revealed an unprecedented level of structural detail of the sorting platform; however, many of the structure-function relationships within this complex remain enigmatic. Here, we report on improved cryo-electron tomographic approaches to enhance the resolution of the *Shigella* T3SA sorting platform (at ≤2 nm resolution) done in concert with biochemical and genetic methods to define the sorting platform interactome and interactions with the T3SA inner membrane ring (IR). We observed that the sorting platform consists of “pods” with 6-fold symmetry that interact with the Spa47 ATPase via radial extensions comprising MxiN. Most importantly, MxiK maintained an interaction with the IR via specific interactions with the cytoplasmic domain of the IR protein MxiG (MxiG⁴), which is a noncanonical forhead-associated domain, and MxiK has an elongated structure that interacts with the IR via MxiG⁵. T4 lysozyme-mediated insertional mutagenesis of MxiK revealed its orientation within the sorting platform and enabled disruption of interactions with its binding partners, which abolished sorting platform assembly. Finally, a comparison with the homologous interactions in the *Salmo-
nella* T3SS sorting platform revealed clear differences in their IR-sorting platform interfaces that have possible mechanistic implications.

Type III secretion systems (T3SSs)⁴ are essential virulence components for a wide range of Gram-negative bacterial pathogens (1, 2). The *Shigella flexneri* T3SS allows for the delivery of host altering effector proteins that can lead to uptake and apoptosis/pyroptosis in macrophages and entry into intestinal epithelial cells, where it multiplies within the cytoplasm (3, 4). The T3SS apparatus (T3SA) is comprised of four distinct components: 1) the needle tip complex, which directs the formation of a translocon pore in the host cell membrane; 2) the needle, which is a polymer of the protein MxiH; 3) the envelope-spanning basal body; and 4) the cytoplasmic sorting platform (SP) (5–8). The SP was originally described as a complex in *Salmo-
nella* that included OrgA, SpaO, and OrgB (8), which are equivalent to MxiK, Spa33, and MxiN, respectively, in *Shigella* (7). Our understanding of the composition and organization of the SP has evolved with recent cryo-electron tomography (cryo-ET) work using minicells of *Shigella* and *Salmonella* (5, 6). The SP provides the energy needed for effector protein delivery via the Spa47 ATPase (considered here as part of the SP) in *Shigella* and presumably through harnessing the difference in electrical potential across the bacterial inner membrane (proton motive force) (9, 10). A key interface between the basal body and the SP in *Shigella* involves an interaction between the inner membrane ring (IR) formed by MxiG and MxiJ via the cytoplasmic domain of MxiG (MxiG⁵), comprised of residues 1–126 (11–13). An interesting feature of this interface is a switch from the 24-fold

⁴The abbreviations used are: T3SS, type III secretion system; T3SA, type III secretion apparatus; IR, inner membrane ring; T4L, bacteriophage T4 lysozyme; lpA, invasion plasmid antigen; Mxi, major exporter of Ipa proteins; Spa, surface presentations of antigen; BLI, biolayer interferometry; SP, sorting platform; cryo-ET, cryo-electron tomography; FP, fluorescence polarization; MxiG⁴, cytoplasmic domain of MxiG; FHA, forhead-associated domain; BACTH, bacterial adenylate cyclase two-hybrid; Amp, ampicillin; Kan, kanamycin; IMAC, immobilized metal affinity chromatography; AEBSF, 4-benzenesulfonyl fluoride hydrochloride; LDAO, N,N-dimethyl-dodecylamine N-oxide; PDB, Protein Data Bank; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.
symmetry of the IR components (14) to a 6-fold symmetry for the “pods” that make up the SP (5, 6). Based on the work presented here on Shigella and recent similar work on Salmonella, this symmetry change appears to be handled differently in different organisms (see “Discussion”).

As presented here, critical pieces of the Shigella SP include the central hexameric ATPase (Spy47) (9), which is connected to the MxiA export ring of the basal body (15) via a stalk protein (Spy13) (16). Spy47 also interacts with MxiN (17), which forms radial “spokes” (6) that connect the ATPase with the major SP pod density that is comprised of multiple copies of Spa33 (6, 12). How these structures collectively interface with the IR, presumably through direct interaction with MxiG\(^C\), remains unresolved and is a focus of this investigation.

Structural studies of the in situ cytoplasmic SP of S. flexneri became possible using cryo-ET analysis of minicells generated through overexpression of the Escherichia coli cell division genes ftsQ, ftsA, and ftsZ (6). In a mxiN null mutant, remnants of the pods could still be seen; however, the inner portions of the SP (Spy47 and Spa13) were missing (6). In contrast, for a spa33 null mutant, the entire SP was missing, suggesting that Spa33 is required to maintain this complex in association with the IR. Meanwhile, MxiN anchors and ensures proper placement of Spy47 in line with MxiA. The missing component at this point appears to be MxiK, which has been shown to co-purify with the other SP components and is essential for Shigella T3SS function (18).

In this study, we present new high-resolution in situ structures confirming that MxiK is the linker between the Spa33 pod protein and the MxiG IR protein, with both interactions being required for SP assembly. Interestingly, the interface between MxiK and MxiG is different from that seen for the homologous proteins (OrgA and Prgh, respectively) in Salmonella. We also demonstrate the proposed protein-protein interactions contributing to Shigella SP assembly in vitro. In particular, we focus on a specific interaction between MxiGC and MxiK and the importance of this interaction in SP assembly and function. Our findings show that the SP can accommodate added densities at multiple sites on MxiK, and such densities are useful in spatially orienting MxiK with respect to its two binding partners (Spy33 and MxiG). The nature of the MxiGC residues needed to promote functional contact with MxiK do not appear to be related to the previous suggestion that it behaves as a phosphopeptide binding forhead-associated (FHA) domain (13). Canonical FHA domains are \(\beta\)-sandwich structures that bind phosphorylmoieties in their protein binding partners; however, it is now clear that there are phosphorylation-independent roles for FHA domains in protein-protein interactions (12). In the T3SS, we propose that it is the FHA domain of MxiG that is responsible for its phosphopeptide-independent interactions with the MxiK adaptor protein of the SP. Furthermore, it is likely that the flexibility of the MxiG linker that connects MxiGC with its transmembrane helix contributes to the dynamics that guide type III secretion in Shigella, but for an unknown reason, the linker in MxiGC of Shigella gives rise to a much different interface than does its counterpart in Salmonella.

### Results

#### Architecture of the T3SA sorting platform in Shigella minicells

Cryo-ET analysis was used to generate the high-resolution in situ structure of the intact Shigella T3SA (Fig. 1 and Fig. S1). In this structure, the cytoplasmic and periplasmic domains of MxiG (outer component of the IR) are visible on opposite sides of the inner membrane, revealing an evenly spaced 24-fold symmetry for both (Fig. 1 (B and C) and Fig. S1 (B and C)), which fits with previous observations by Schraidt and Marlovits (14). In contrast, the density immediately beneath MxiGC, which we propose is MxiK (6), appears to have 6-fold symmetry with densities evenly spaced around the export gate (MxiA) of the basal body. In addition, the 6-fold symmetry of Spa33 (the major pod component) and MxiN (radial spokes connected to the Spa47 ATPase) are shown in Fig. S2. The putative MxiK densities are bi-lobed, perhaps suggesting a two-domain composition (Fig. 1D and Fig. S1D). An interesting feature here is that the interface is markedly different from that seen for Salmonella, where PrghC (equivalent to MxiGC) domains cluster into tetramers where they interact with OrgA (equivalent to MxiK) (5). This clustering is not present in the Shigella SP.

In parallel experiments, the in situ structure of the T3SA from a Shigella mxiK null mutant was determined (Fig. 2A). The absence of MxiK leads to complete loss of the SP, although the basal body and its export channel (MxiA) are still visible. All extracellular T3SA structures, including the needle and needle tip complex, are completely absent for the mxiK null mutant. These mutants had lost all virulence-related functions (e.g. steady-state secretion of invasion plasmid antigens, contact-mediated hemolysis and invasion of cultured cells), but these were restored to WT levels after complementation with the plasmid pWPsf4-mxiK, which allows expression of WT mxiK (see below).

Upon zooming in on a single pod from the WT T3SA (Fig. 2B), multiple densities can be seen within the cross-section of a single pod (Fig. 2, C and D). When examined from the front and after further refinement to a resolution of 1.8 nm (Fig. 2E), the density of what is presumed to be MxiK is apparent with what may be two domains (compare Fig. 1D and Fig. 2E). Surface renderings for the pods depicted in Fig. 2 (B–E) are shown in Fig. 2 (F–H), respectively, and these more clearly define the densities of a single pod from the side (Fig. 2G) and front face (Fig. 2H).

#### The protein-protein interactions that make up the Shigella T3SA sorting platform

We used a bacterial adenylate cyclase two-hybrid (BACTH) system (19, 20) to tentatively identify the protein–protein interactions that define the SP in vitro (Fig. S3 and Table 1). In these analyses, no interactions were identified for Spa33 and MxiGC, as was previously suggested to occur for peptides derived from Spa33 (Table 1) (13). Care should be taken in interpreting such a negative result, however, because a potential caveat of BACTH analyses is that they require establishing a proper alignment for the T18 and T25 domains of CyaA, which may not occur for some interacting partners. In fact, BACTH experiments failed to identify interactions between Spa47 and MxiN,
although this interaction is widely known to occur (17). Unlike the previous study suggesting a Spa33-MxiGC interaction, however, we could not demonstrate an interaction for peptides derived from Spa33 (including phosphorylated peptides) with MxiGC using biolayer interferometry (BLI) analyses with MxiGC immobilized onto sensor tips. Instead, our BACTH analyses indicated that MxiK associates with MxiGC (Table 1 and Fig. S3); however, this interaction was slow to appear using the BACTH system. Therefore, to confirm this interaction is real, we tested the ability for peptides from a library spanning the length of MxiK to bind to MxiGC using BLI. Indeed, three peptides from this library did show specific binding to MxiGC, and these all localized to a region within the N-terminal half of MxiK (Table 2). It is important to note, however, that the interaction for a peptide spanning residues 30–60 of MxiK, for example, was somewhat weak, with a dissociation constant in the micromolar range (Table 2 and Fig. S4).

To complement the BLI analysis, a peptide consisting of MxiK residues 50–60 with two extra lysines at the N terminus was labeled with FITC so that its binding to MxiGC could be monitored using fluorescence polarization (FP). As shown in Fig. 3, the FITC-labeled peptide was bound by MxiGC to confirm that peptides from this region of MxiK can be bound by MxiGC; and, in this case, the observed dissociation constant was much lower (0.15 μM) than those observed using BLI. Nevertheless, the interaction appeared to be specific because non-
MxiG and MxiK guide sorting platform assembly

Figure 2. MxiK is required for formation of the sorting platform and forms an interface with MxiG. A, cryo-ET image of the S. flexneri T3SA for a mxiK null mutant. Relative to the apparatus from WT bacteria (B), the entire sorting platform and the extracellular needle are absent, and the central channel is blocked within the basal body. The cytoplasmic MxiA export gate is still visible in this structure. C, zoom view of the single “pod” located in the boxed region of B (WT T3SA). The boundaries between the domains of the pod are not well-defined, partly because of the flexibility of the pod. In D, the pod is shown after further refinement with several densities becoming apparent. To better define the pod structure, a perpendicular view of this pod is shown in E, in which additional boundaries and protein-protein interfaces become visible. F, a surface rendering of the image shown in B with the darkened region representing a single pod. That pod’s rendering is enlarged in G (equivalent to D), where a single small density interfaces with the bacterial inner membrane (containing the IR). A larger multidomain density makes up the bulk of the pod. H then shows a perpendicular representation of the pod (equivalent to the image in E). When bacteriophage T4L was fused to the C terminus of MxiK, it retained its WT activity, and the resulting added density could then be seen on the inside of the SP density nearest the IR (shown as the green structure in J, which is equivalent to the rendering in G). MxiK is thus indicated by the orange structure shown here. J then is a perpendicular image of the rendering shown in I with the T4L density masked by the outer face of MxiK. Based on the SP interactome reported in Table 1, the larger density shown in yellow is a complex of multiple Spa33 proteins. CM, cytoplasmic membrane; OM, outer membrane.

Table 1
Sorting platform interactome

BACTH analysis was used to determine interactions between key sorting platform components. Positive interactions were determined by X-gal cleavage on LB agar plates or maltose utilization on MacConkey agar plates. Positive interaction: +, negative interaction: −, ND, not determined.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MxiG&lt;sup&gt;C&lt;/sup&gt;</th>
<th>MxiK</th>
<th>Spa33</th>
<th>MxiN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MxiG&lt;sup&gt;C&lt;/sup&gt;</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MxiK</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spa33</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MxiN</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2
Interaction of MxiK peptides with MxiG<sup>C</sup> based on BLI analysis

Peptides were used in a BLI assay to determine their ability to bind to MxiG<sup>C</sup> immobilized onto sensors via an N-terminal His<sub>6</sub> tag. NA, not applicable.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Peptide no.</th>
<th>Specific binding to MxiG&lt;sup&gt;C&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–30</td>
<td>P5</td>
<td>No</td>
</tr>
<tr>
<td>15–45</td>
<td>P3</td>
<td>No</td>
</tr>
<tr>
<td>30–60</td>
<td>P4</td>
<td>Yes</td>
</tr>
<tr>
<td>45–75</td>
<td>P6</td>
<td>Yes</td>
</tr>
<tr>
<td>50–70</td>
<td>P7</td>
<td>Yes</td>
</tr>
<tr>
<td>60–80</td>
<td>P8</td>
<td>No</td>
</tr>
<tr>
<td>60–90</td>
<td>P9</td>
<td>No</td>
</tr>
<tr>
<td>70–90</td>
<td>P12</td>
<td>Weak</td>
</tr>
<tr>
<td>75–105</td>
<td>P10</td>
<td>Not soluble&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>90–120</td>
<td>P13</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>105–135</td>
<td>P11</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>130–160</td>
<td>P12</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>145–175</td>
<td>P13</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fluorescence polarization analysis of a fluorescein-labeled MxiK peptide 51–60 was also performed (see Fig. 3).

<sup>b</sup> Peptide P10 was not soluble in solution, which prevented determining its ability to bind to MxiG<sup>C</sup>.

transient or dynamic one. We also observed a similar interaction between the homologous pair (PscD<sup>C</sup> and PscK) from Pseudomonas aeruginosa.<sup>5</sup> Taken together, these data strengthen the argument that MxiG and MxiK form the interface between the SP and the IR of the basal body.

Once all of the SP components had been tested for their ability to interact with all of the other components using the BACTH analysis, the protein-protein interactions that were clearly established included MxiGC-MxiK, MxiK-Spa33, and Spa33-MxiN (Table 1). Although not observed via BACTH analysis, we also know that a MxiN-Spa47 interaction maintains the ATPase at the core of the SP and controls the ATPase activity (17). This suggests a working model outlining the architecture for the Shigella SP starting at the IR as being MxiG (IR)—MxiK (IR/SP adaptor protein)—Spa33 (the major density of the pods)—MxiN (spoke protein)—Spa47 ATPase. Convention would then have it that Spa13 links Spa47 with the major export protein MxiA of the basal body (16). In addition to these interactions, MxiN was able to interact with itself (presumably forming a dimer), and Spa33 was able to interact with itself. Indeed, Spa33 comprises a large, multicomponent density that ultimately creates the bulk of the pods, suggesting a significant role for this protein in pod formation and SP function. We have

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Figure 3. MxiK peptide binding to MxiGC measured using FP. A peptide comprised of MxiK residues 51–60 (YDLNCDIEPL) was generated with an additional two lysine residues placed at the N terminus to permit labeling with FITC. Left, the labeled peptide (100 nm) was incubated with increasing concentrations of unlabeled MxiGC. This is a plot of the resulting change in the FP (given as millipolarization units (mP)) value (n = 6) with S.D. (error bars). Right, the ability of the labeled peptide (100 nm) to bind to 100 nm MxiGC was monitored in the presence of increasing concentrations of the 10-amino acid peptide representing residues 51–60 of MxiK.

Figure 4. Mutations in MxiG and MxiK that block T3SS activity result in loss of SP assembly and formation of the external needle. A library of T4L insertion mutants was generated for MxiK, and they were expressed in a Shigella mxiK null mutant. The ability of these bacteria to carry out contact-mediated hemolysis (A) and secretion of the translocator protein IpaB (B) was then tested. In parallel, a mutant mxiG gene encoding Ala in place of residues 61–66 and 81–85 was generated (mxiG61–66/81–85Ala). This gene was then expressed in a Shigella mxiG null mutant, and contact-hemolysis (A) and IpaB secretion were tested (B). In parallel, S. flexneri minicells expressing mxiG61–66/81–85Ala were examined by cryo-ET. These minicells (C) fail to form the external T3SA needle, and SP formation does not occur (E). The same result is seen when S. flexneri minicells are making MxiK-T4L-136 in place of WT MxiK (D and F, respectively). Error bars, S.D.
previously shown that spa33 null mutants completely lack pods much like the mxiK mutant shown in Fig. 2A, whereas mxiN null mutants still display the remnants of pods while completely lacking the central Spa47 ATPase.

**MxiK can accommodate additional molecular densities at multiple sites throughout its structure**

To better understand the positioning of MxiK within the SP, we generated a library of MxiK insertion mutants so that the added density that could be tracked by cryo-ET methods. We fused bacteriophage T4L at the termini and within predicted loops of MxiK. T4L was used for this purpose because its N and C termini are very close together (≤1 nm), which minimizes the likelihood that insertion into internal loops would disrupt MxiK’s overall folding (21). Surprisingly, in nearly all cases when the MxiK-T4L insertion mutant was used to complement a *Shigella* mxiK null mutant, a substantial portion of *Shigella’s* *in vivo* virulence activities (IpaB secretion and contact-mediated hemolysis) were restored (Fig. 4, A and B). Because the addition of T4L at the C terminus caused no loss of secretion or hemolysis activities, and because this protein could be purified and shown to bind MxiG in vitro, it was used to determine where the added density might appear within the SP by using cryo-ET analysis of minicells. As shown in Fig. 2 (I and J), the extra density at the MxiK C terminus appears on the inside face of the SP component located adjacent to the inner membrane (associated with the IR). To better indicate the added density, a difference map was generated to confirm the location of T4L (Fig. S6). These data are in agreement with BACTH data and confirm the position of MxiK as an adaptor protein that provides the interface between the SP (MxiG) and the T3SA basal body. They also provide information on MxiK’s orientation with the C terminus facing inside the SP “cage.”

**Generation of mutants that fail to allow for sorting platform assembly and activity**

Unlike with T4L insertional mutagenesis of MxiK, internal insertions of GFP were not able to restore SP assembly or function. This is not surprising because the N and C termini of GFP are far apart and would be expected to disrupt MxiK folding. However, two T4L insertion mutants also failed to restore *Shigella* virulence activities, and this prompted us to specifically look for mutations that abolish SP formation to better characterize the key protein-protein interactions needed for assembly. First, we chose to target the FHA domain β-structure of MxiG, for which it seemed reasonable that loop structures would have an important functional role related to its association with the SP via interactions with MxiK. Prior studies identifying FHA domain similarities suggested that residues Arg-39, Ser-61, and Ser-63 might be functionally important (13); however, Lea’s group showed that this was not the case (12). In fact, we found that the entire loop comprised of residues 61–66 could be replaced with all Ala residues without loss of function. Likewise, when a neighboring loop (residues 81–85) was replaced with five Ala residues, type III secretion was not perturbed; however, when both loops were changed to strings of Ala, type III secretion was completely abrogated (Fig. 4 (A and B); see Fig. S7). When BACTH analysis of the MxiGβ version of this mutant was completed, it appeared to have lost its ability to interact with MxiK. When minicells of this mutant were examined by cryo-ET, the basal body could be seen, but the entire SP was missing, and no extracellular components of the T3SA were present (Fig. 4, C and E). The fact that the T3SA basal body formed correctly for the MxiG<sub>61–66/81–85</sub>Ala mutant (Fig. 4E) indicated that this mutation within the MxiG N-terminal domain did not interfere with its ability to be recruited into the T3SA. Thus, this mutation located within the N terminus of MxiG did not cause loss of T3SS function due to global misfolding of the protein in *Shigella*.

Because a mutation in MxiG that prevents its interaction with MxiK abolishes SP assembly, we moved forward with a panel of MxiK-T4L insertion mutants to see whether any of those had lost their ability to restore virulence activities (Fig. 4, A and B). The goal here was to determine whether key protein-protein interactions could be blocked by the added T4L density. We identified two MxiK-T4L insertion mutants (at positions 25 and 136) that had greatly reduced or no ability to restore secretion activity to a mxiK null *Shigella* strain (Fig. 4, A and B). We moved forward with the MxiK-T4L-136 mutant. To assess whether the loss of activity could be explained by protein misfolding, we examined the thermal unfolding profile for MxiK-T4L-136 and compared it with that of MxiK-T4L-C, which is fully active. These mutants had CD spectra that were virtually identical (Fig. S8), but more importantly, both proteins had similar thermal stability, suggesting that these T4L insertions had not compromised MxiK’s structural stability (Fig. S8). The observation that both MxiK-T4L-C and MxiK-T4L-136 could interact with MxiG<sub>β</sub> in BACTH analyses (alluded to below) also suggested that each was similarly folded.

When MxiK-T4L-136 was examined in minicells using cryo-ET, the T3SA basal body appeared normal, but the entire SP and all extracellular components were missing (Fig. 4, D and F). This is identical to what was seen for a mxiK null mutant and for the above MxiG Ala mutant; however, when BACTH analysis was used, the MxiK-T4L-136 interaction with MxiG<sub>β</sub> could still be observed. In contrast, when it was examined for its alternative protein-protein interaction (with Spa33), no BACTH signal was observed. This finding suggests that MxiK’s role as an adaptor protein suggests that it serves as a bridge between MxiG and Spa33 and any disruption of either interaction completely eliminates SP assembly. This would be consistent with MxiK and Spa33 interacting within the SP assembly as a stable dimeric complex or as a single functional unit.

Because a peptide representing MxiK residues 51–60 associates with MxiG<sub>β</sub> (Table 2 and Fig. 3), we also selected this region for mutation analysis. Conversion of the 10-amino acid region from 51 to 60 to all Ala residues completely eliminated MxiK’s ability to restore secretion to a mxiK null *Shigella* strain, as did a five-residue Ala replacement from 51 to 55 (Fig. 5). When pared down to a single Ala substitution (MxiK<sub>Leu53Ala</sub>), the mutated MxiK was still unable to direct type III secretion; however, a MxiK<sub>Asp56Ala</sub> mutant turned out to be fully active (Fig. 5). When the 51–55 Ala substitution (along with the MxiK<sub>Leu53Ala</sub>) was introduced into MxiK-T4L-C and the secondary structure analyzed by CD spectroscopy, it appeared to be folded correctly (Fig. S8). When used for BACTH analysis,
the 51–55 Ala mutant’s ability to associate with MxiG\(^{C}\) was eliminated; however, its ability to interact with Spa33 was also lost. MxiK\(^{Leu53Ala}\) also appeared to have lost its ability to interact with MxiG\(^{C}\) and, surprisingly, its ability to interact with Spa33. In contrast, the active mutant MxiK\(^{Asp56Ala}\) was able to associate with both MxiG\(^{C}\) and Spa33, as might be expected. Whereas these data support the proposition that amino acids within residues 51–60 of MxiK are important for its ability to serve as an adaptor between the T3SA basal body and SP, they are complicated by the fact that Spa33 binding is also lost. This is puzzling because CD analysis does not suggest that the protein is misfolded by these mutations.

**The sorting platform within the context of the overall Shigella T3SA**

Fig. 6 (A and B) shows a surface rendering of the entire *Shigella* T3SA with assignments for all the SP components. The cross-section shown in Fig. 6B more clearly shows the positions of the MxiN spoke protein, the Spa47 ATPase, and the MxiA export gate. It also shows the alignment of Spa33 (yellow) and MxiK (orange) with MxiG\(^{C}\) and the outer portion of the periplasmic domain of MxiG (MxiG\(^{P}\)) (both shown in light blue). When viewed from above (Fig. 6C), the alignment of the IR comprised of 24 nearly contiguous copies of MxiG\(^{C}\) directly over the MxiK/Spa33 pods having evenly spaced 6-fold symmetry is clear. Although the MxiG\(^{C}\) ring is slightly offset from the pods, the ability for MxiK to simultaneously contact MxiG\(^{C}\) and Spa33 is evident. The arrangement of these proteins relative to the Spa47 ATPase, which is connected to the pods via MxiN and the MxiA export gate via Spa13 gives the immediate impression of having the capacity of a rotary motor that could drive effector protein secretion. This has also been suggested by others based on the structure of the central hexameric ATPase of the T3SS (22). Such a mechanism would imply that there are tightly controlled dynamic interactions occurring within the SP, particularly where the SP interfaces with the IR via MxiK.

**Discussion**

Here we demonstrate key interactions between the proteins required for assembly of the *Shigella* T3SA SP using complementary *in vivo* and *in vitro* analyses. The cytoplasmic FHA domain of the T3SA basal body IR protein MxiG (referred to as MxiG\(^{C}\)) provides the foundation upon which SP assembly occurs. We propose here that SP assembly first requires a productive interaction between MxiG\(^{C}\) and the SP adaptor protein MxiK. Meanwhile, formation of the MxiK-Spa33 complex gives rise to the stabilizing “pods” that allow recruitment of the remaining SP components, including the Spa47 ATPase that is held in place via MxiN interactions with Spa33. Mutations that destabilize key interactions involving MxiK completely disrupt SP assembly and these interactions are summarized in Fig. 7. A mutation in MxiK\(^{C}\) that eliminates its ability to associate with MxiK results in a complete loss of SP assembly and all T3SS functions. Likewise, the addition of a new density to MxiK that blocks its ability to associate with Spa33 eliminates T3SS functions and SP assembly (Fig. 7). Perhaps not surprising then, some mutations within a region of MxiK corresponding to a peptide that binds to MxiG\(^{C}\) (but not Spa33), appear to interfere with the MxiK-MxiG\(^{C}\) interaction, and this eliminates T3SS functions in *Shigella*. Unexpectedly, however, these inactivating mutations also interfere with the MxiK-Spa33 interaction, and this does not appear to be due to MxiK misfolding. Certainly, the complexities of the MxiK-Spa33 interaction warrant further study.

Although the structure of MxiK (or any of its homologues from other systems) is not known, we can deduce from the data presented here that its C terminus faces inside of the SP assembly and the region around residue 136 is oriented downward toward the Spa33 complex. The change in symmetry at the MxiG-MxiK interface would fit with a mechanism involving structural changes at this interface, which would accommodate the interaction. In fact, this has been observed for the homologous interaction in the *Salmonella* T3SA between PrgH and
OrgA (8). In Salmonella, PrgH\(^{C}\) is remodeled into clusters of four at the point where they interact with OrgA (5). This can only be accommodated by flexibility in the linker between PrgH\(^{C}\) and the PrgH transmembrane helix. Indeed, this flexibility becomes evident when the density map of a purified Salmonella injectisome (23, 24) is fit to the \textit{in situ} model of the Shigella T3SA (Fig. 8). For the purified injectisome from Salmonella, PrgH\(^{C}\) is completely imbedded in the cytoplasmic membrane when overlaid onto the \textit{Shigella in situ} model. In other words, it is not present where the MxiGC density is observed. When moved downward to accommodate the inner membrane, PrgH\(^{C}\) would still form a ring that is smaller than that formed by MxiGC \textit{in situ} (Fig. 8H), thus suggesting that PrgH\(^{C}\) can move to accommodate OrgA binding.

Such remodeling may be important for functional dynamics that are driven by the T3SS ATPase or possibly by the promotive force (10, 25, 26). In contrast to PrgH\(^{C}\) in the \textit{in situ} Salmonella T3SA model, however, no clustering is observed for MxiG\(^{C}\), which remains evenly spaced on the inner face of the cytoplasmic membrane (see Fig. 1 and Fig. S1). Perhaps this is due to the slightly shorter linker between MxiG\(^{C}\) and its transmembrane helix; however, this makes it difficult to conclude that the remodeling is mechanistically important, at least in the Shigella system. Moreover, when 11 residues were removed from the \textit{Shigella} MxiG linker region, there was no decrease in secretion activity, suggesting that even further restriction of the potential range of MxiGC movement is not detrimental to function.

It is noteworthy that in a \textit{mxiK} null mutant (Fig. 2A), the position of MxiG\(^{C}\) becomes difficult to pinpoint, which does fit with its ability to remodel within the \textit{Shigella} cytoplasm in the absence of an intact SP. Nevertheless, it is not clear that major remodeling occurs for MxiG\(^{C}\) within the active T3SA of \textit{Shigella}, and eliminating almost half of the linker between the

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**Figure 6.** Surface rendering of the proposed placement of the IR and SP components. A, rendering of the sorting platform indicating the positions of MxiG (MxiG\(^{G}\) above the inner membrane and MxiG\(^{C}\) below it), MxiK, Spa33, MxiN, and Spa47. A cross-section of the complex is shown in B. MxiG\(^{G}\) is very close to the inner membrane, where it interacts with MxiK (arrows). This view better depicts MxiN and Spa47 with the export gate (MxiA) found inside the MxiG\(^{G}\) ring. The homologous models of MxiG\(^{G}\) (PDB code 6DU2), MxiG\(^{C}\) (PDB code 3J1W), basal body (PDB code 6DV3), rod (PDB code 6DWB), and socket (PDB code 6F2D) were fitted into the structure. C, top view of the MxiG\(^{G}\) docking model.

**Figure 7.** Summary of MixK interactions with MxiG\(^{C}\) and Spa33. WT MxiK and MxiK-T4L-C are able to restore virulence activities to a \textit{mxiK} null \textit{Shigella} mutant. In contrast, mutations that disrupt MixK association with MxiG\(^{C}\) or Spa33 \textit{in vitro} fail to restore contact-hemolysis or IpaB secretion activities to \textit{Shigella}. Interestingly, a single substitution mutation at MixK position 53 (MixK\(^{Leu53Ala}\)) eliminated hemolysis and secretion activity, and this was predicted to be due to loss of an interaction with MxiG\(^{C}\); however, this mutant was also defective in Spa33 binding \textit{in vitro}.
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Figure 8. Remodeling of MxiG<sup>C</sup> ring in <i>S. flexneri</i>. A, a density map of the purified needle complex from <i>Salmonella</i> (gray color, EMD-1875) was fitted into the <i>in situ</i> T3SA structure from <i>Shigella</i>. The corresponding atomic model of the purified needle complex from <i>Salmonella</i> was fitted into the <i>Shigella</i> T3SA structure in a side view (B) and a cross-section view (C), respectively. In C, the cytoplasmic domain of the <i>Salmonella</i> PrgH cytoplasmic domain (PrgH<sup>2</sup>) of the inner ring 2 (IR2), equivalent to MxiG<sup>C</sup>, is completely embedded in the cytoplasmic membrane of the <i>Shigella in situ</i> T3SA model. In fact, it takes about a 5-nm shift of PrgH<sup>2</sup> for it to be relocated underneath the cytoplasmic membrane in D and E. In addition, the original PrgH<sup>2</sup> ring (gray) is smaller than the IR2 density actually formed by the MxiG<sup>C</sup> density seen in the <i>Shigella in situ</i> model, as shown in blue in F–H. G and H, a central cross-section and a top view of F, respectively.

MxiG<sup>C</sup> FHA domain and the MxiG transmembrane helix does not reduce secretion efficiency. Other changes within the T3SA structure for the <i>mxiK</i> null mutant include a possible change in the position of the export gate (MxiA) and blockage at multiple points within the T3SA central channel (Fig. 2, A and B), and this also appears to be the case for a <i>spa33</i> null mutant (6).

When everything is considered, a model in which highly dynamic protein-protein interactions occur at the SP/IR interface can be envisioned. Dynamics within the SP of the <i>Yersinia</i> T3SA involving the MxiK and Spa33 homologues YscK and YscQ, respectively, have been described (27, 28). Further exploration of the IR-MxiK interface and the identification of the dynamics of the protein-protein interactions occurring across multiple T3SA from diverse organisms will be needed to generate a consensus model for a mechanism of action for the T3SS SP.

Experimental procedures

<i>Strains</i>

<i>S. flexneri</i> serotype 5a (M90T) (29) and a <i>mxiK</i> null mutant strain were from John Rohde (Dalhousie University, Halifax, Canada). Minicells of WT and Δ<i>mxiK</i> <i>S. flexneri</i> were generated by introducing plasmid pBS58, which continuously expresses the <i>E. coli</i> cell division genes ftsQ, ftsA, and ftsZ from a low-copy, spectinomycin-resistance plasmid (30). Bacterial cultures were grown overnight at 37 °C in trypticase soy broth, and fresh cultures were prepared from a 1:100 dilution and then grown at 37 °C to late log phase. Spectinomycin (100 μg/ml) was added for selection of pBS58, 50 μg/ml kanamycin was added for selection of the parent <i>mxiG</i> or <i>mxiK</i> null mutant, and 100 μg/ml ampicillin was added for selection of the plasmid expressing mutant <i>mxiG</i> or <i>mxiK</i>. To enrich for minicells, the culture was centrifuged at 1000 × g for 5 min to remove the large cells, and the supernatant fraction was further centrifuged at 20,000 × g for 10 min to collect the minicells.

<i>Cloning of MxiK-T4L insertion mutants</i>

The T4L gene was amplified by PCR using primers containing additional nucleotides so that the T4L gene would have linker regions at the 5′ and 3′ ends encoding Gly-Ser. The T4L coding sequence (the catalytically inactive mutant E11A) was then inserted at the N or C terminus of MxiK, as well as after amino acids 25, 55, 60, 101, 136, and 145, using ligation-independent cloning with WT MxiG and the pT7HMT vector as a template (21). Each of the resulting insertion mutants was used as a template for cloning into the NdeI/BamHI-digested <i>pWPsf4</i> plasmid vector for sequence confirmation (31). To construct plasmids for the pT7HMT expression vector for phenotypic analysis and for BACTH experiments (19, 20), <i>mxiK</i> mutant genes in <i>pWPsf4</i> were used as templates. Recombinant plasmids were introduced into a <i>mxiK</i> null mutant of <i>S. flexneri</i> by electroporation.

<i>Cloning of mxiG<sup>61–66/81–85Ala</sup> in pWPsf4</i>

For MxiG with Ala residues replacing residues 61–66 and 81–85 (<i>mxiG<sup>61–66/81–85Ala</sup></i>), WT <i>mxiG</i> cloned into <i>pWPsf4</i> (31) was used as the template, and primers for inverse PCR were designed so that the PCR products contain 61–66 Ala encoding nucleotides at 5′ and 3′ ends. After purification of the resulting PCR product, In-Fusion Enzyme Premix (Takara Bio,}
Inc.) was used to ligate the 5’ and 3’ ends of the plasmid. For mxiG\textsubscript{61–66/81–85Ala}, the mxiG\textsubscript{61–66Ala} plasmid was used as the template. Primers were designed to introduce Ala substitutions in the 81–85 region, and the same procedure was used as for the cloning of mxiG\textsubscript{61–66Ala}.

**Overnight steady-state secretion assay**

The secretion activity of T3SS was tested by detecting IpaB levels in the overnight bacterial cultures (31). Colonies of S. flexneri and its various mutants were taken from trypticase soy agar plates containing ampicillin (Amp) and kanamycin (Kan) and inoculated into trypticase soy broth with Amp and Kan. The bacterial cultures were grown overnight at 37 °C. The supernatant fractions were collected by centrifugation, and the proteins in the supernatant fraction were precipitated in 10% TCA and then centrifuged at 10,000 rpm for 15 min at 4 °C to collect the precipitated protein. The protein was washed with TCA and then centrifuged at 10,000 rpm for 15 min at 4 °C to collect the precipitated protein. The protein was washed with 5% TCA and ice-cold acetone. After evaporating the acetone, the proteins were resuspended in 400 μl of 10 mM phosphate, pH 7.4, 150 mM NaCl (PBS) and used for the immunoblot analysis. For the immunoblot, a 10% SDS polyacrylamide gel electrophoresis was used to separate the proteins for blotting onto nitrocellulose membranes. Rabbit anti-IpaB serum and secondary donkey anti-rabbit IgG with an IR tag (LI-COR Biosciences, Lincoln, NE) were used to detect IpaB. Densitometry was used to quantify the relative amounts of IpaB secreted by each strain.

**Contact-mediated hemolysis**

The ability for Shigella to insert functional translocons into target cell membranes was tested by contact-mediated hemolysis (31). Different S. flexneri strains were grown on trypticase soy agar plates containing Amp and Kan. Colonies formed on these plates were inoculated into trypticase soy broth with Amp and Kan individually and grown at 37 °C until mid-log phase. A 10-ml aliquot of bacterial culture was centrifuged at 4000 rpm for 10 min at 30 °C, and the bacterial pellets were resuspended in PBS to an equivalent bacterial density for each strain. Defibrinated sheep red blood cells (Colorado Serum Co., Denver, CO) were washed in 40 ml of PBS and then resuspended in 3 ml of PBS. The resuspended bacteria (50 μl) were added to 50 μl of washed red blood cells on 96-well plates. The plate was centrifuged at 3500 rpm for 15 min at 30 °C and then incubated at 37 °C for 1 h. The cell mixtures were resuspended in 100 μl of cold PBS, and the plate was centrifuged at 3500 rpm for 15 min at 10 °C to obtain supernatants from the mixture of red blood cells and bacterial samples. The supernatants were transferred into new 96-well plates, and the concentration of released hemoglobin was measured by absorbance at 545 nm.

**Recombinant protein expression and purification**

Tuner (DE3) E. coli was transformed with pT7HMT expression vectors into which each of the generated mxiK mutant genes had been inserted (32). These expression strains were inoculated and grown at 37 °C overnight. A 10-ml aliquot of the overnight cultures was added to 1 liter of lysogeny broth with Kan. The bacterial cultures were grown until the A\textsubscript{600} reached 0.6. Generation of the recombinant protein with a His\textsubscript{6} tag was then induced with 0.25 mM isopropyl β-D-1-thiogalactopyranoside at 16 °C overnight. Bacterial pellets were resuspended in binding buffer for nickel resin affinity chromatography (IMAC) with the protease inhibitor AEBSF (Sigma) and lysed by ultrasonication. The bacterial lysate was centrifuged at 12,000 rpm for 30 min at 4 °C to obtain insoluble pellets. The insoluble pellets were washed by the binding buffer twice and then resuspended in the binding buffer with AEBSF and 0.1% N,N-dimethyl-dodecylamine N-oxide (LDAO). The suspension was incubated at 4 °C and then centrifuged at 12,000 rpm for 30 min at 4 °C. The recombinant proteins in the supernatant fraction were used for the protein purification by IMAC followed by size-exclusion chromatography on Superdex 75pg (GE Healthcare).

**BLI**

A library of 30-mer peptides overlapping by 15 amino acids covering the entire Shigella MxiK protein sequence was custom-synthesized (PEG Biosciences Inc.). Binding affinity of MxiK peptides to the MxiG cytoplasmic domain (MxiG\textsuperscript{C}) was tested using the Octet Red96 system (ForteBio, Freemont, CA) (33). Binding assays were initiated by capturing His\textsubscript{6}-tagged MxiG\textsuperscript{C} protein (5 μg/ml) on a hydrated nickel-nitrilotriacetic acid biosensor (ForteBio) equilibrated in kinetics buffer (PBS, pH 7.4, 0.01% BSA, 0.002% Tween 20). This was followed by a baseline step in kinetic buffer for 5 min to remove nonimmobilized protein. Once a baseline signal was established, the sensors with immobilized protein were immersed into a solution containing the designated MxiK peptide (association step). Different concentrations of MxiK peptide were tested against the immobilized MxiG\textsuperscript{C}. The dissociation of bound analyte from MxiG\textsuperscript{C} was monitored by mass spectrometer (ForteBio) with an association time of 300 s followed by 300-s dissociation. The sensorgrams from the experiment were fitted with 1:1 binding equations available for interaction from a global curve fit using Data Analysis software version 7.1.0.36 (ForteBio).

BLI was also used to look for the binding of MxiK-T4L-C to MxiG\textsuperscript{C} anchored on the sensor tip. After purifying the His\textsubscript{6}-tagged MxiK-T4L-C, the buffer was changed to PBS containing 0.05% LDAO for storage. For BLI analysis, the protein was added to kinetic buffer (PBS with 0.01% (w/v) BSA and 0.002% (v/v) Tween 20) to a final concentration of 35 μg/ml for mixing with various concentrations of MxiG\textsuperscript{C} (800, 600, 400, 200, and 100 μg/ml). All subsequent steps were similar to those used for measuring the interaction of MxiK peptides with MxiG\textsuperscript{C} except the capturing (loading) time and temperature. To bind enough His\textsubscript{6}-tagged MxiK-T4L-C to the nickel-nitrilotriacetic acid biosensor tip, the capturing step (loading step) was increased to 10 min and then the nonimmobilized proteins were removed by a baseline step with the kinetic buffer for 5 min. The loaded tips were then placed into a solution containing soluble MxiG\textsuperscript{C}. After the association step, sensors were placed into the kinetic buffer to measure the dissociation phase of the interactions. All steps were carried out 20 °C. The background was corrected by
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Subtraction of data from a control sensor having MxiK-T4L-C associated with the sensor tip without MxiGC present. A 1:1 binding equation was used to create the sensorgram from this experiment.

Fluorescence polarization

A peptide comprised of MxiK residues 51–60 (YDLNCDIEPL) was generated with an additional two lysine residues at the N terminus (Synthetic Chemical Core facility, University of Kansas). This peptide (2 mg) was dissolved in 0.1 M sodium carbonate buffer, pH 9.0, and labeled with freshly prepared FITC dye added in a 2-fold molar excess to the peptide. The reaction was incubated in the dark for 2 h at room temperature. Unreacted FITC was removed by separating the reaction components on Sephadex G50 equilibrated with PBS. N-terminal FITC-labeled peptide (100 nm) was mixed with increasing concentrations of MxiGC. FP was measured using a multimode platform microplate reader (Spectra Max i3, Molecular Devices) in black-wall, clear-bottomed microtiter plates. The FP values were determined using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The calculated millipolarization values were plotted as a function of MxiGC concentration to quantify the dissociation constant for the protein-peptide interaction. A competitive binding assay was performed using different concentrations of unlabeled MxiK peptide to test the specificity of the binding.

CD spectroscopy

Secondary structure content was determined on a CD-spectropolarimeter from JASCO (model J-1500) (Jasco Inc., Easton, MD), which was used to measure the molar ellipticity of selected versions of the MxiK-T4L-C mutant (MxiK with T4L fused at its C terminus) (34, 35). These recombinant proteins were prepared in PBS with 0.1% LDAO, which was also used as a blank. For obtaining CD spectra, the ellipticity of the recombinant protein was determined in 0.1-cm path length quartz cuvettes and with scanning from 190 to 260 nm at 10 °C. These data were used to calculate molar ellipticities to normalize for protein concentrations. Secondary structure thermal stability was monitored at 222 nm over a temperature range from 10 to 90 °C. Data were acquired every 2.5 °C, and the temperature ramp rate was 15 °C/h. The thermal transitions were determined by plotting the second derivative of the thermal unfolding curves and identifying the inflection point of each transition (OriginPro 8.6.0).

Cryo-ET data collection and tomogram reconstruction

For Shigella minicells generated from WT, MxiK T4L insertion mutants, and a mxiK null mutant, the frozen-hydrated specimens were imaged at −170 °C using a Titan Krios microscope (FEI Co.) equipped with a field emission gun, Volta phase plate (Thermo Fisher Scientific), energy filter, and direct detection camera (K2 Summit). The microscope was operated at 300 kV, with a pixel size of 3.48 Å at the specimen level. The single-axis tilt series was collected with a Volta phase plate at 0-μm defocus using SerialEM software (36). The cumulative doses of ~50 e−/Å² were distributed over 35 stacks covering an angle range of ~51° to 51° with 3° angle step.

Drift correction for each image stack was done using Motioncor2 (37). Then the tilt series were aligned by IMOD (38, 39), and weighted back-projection reconstructions were done by Tomo3D software (40). 922 tomograms for Shigella WT minicells, 49 tomograms for Shigella MxiG61–66/81–85Ala minicells, and 347 tomograms for the Shigella MxiK-T4L-136 insertion mutant minicells were reconstructed.

Sub-tomogram averaging

The sub-tomograms used for each strain were as follows: 4488 from WT Shigella, 1052 from MxiK T4L insertion mutants, 330 from mxiK null mutant, 91 for MxiG61–66/81–85Ala, and 97 for MxiK-T4L-136. As described previously (5, 6), I3 software (41, 42) was used for sub-tomogram analysis of the T3SA, including the focused refinement. The complete T3SA particles were manually picked from the tomograms as described (43, 44) and then aligned in 4 × 4 × 4 binned sub-tomograms and refined in 2 × 2 × 2 binned sub-tomograms.

After we obtained the average structure of the complete T3SA, a soft mask just including the MxiG portion and adjacent regions was then applied to the T3SA to do the focused refinement for the MxiG portion. Here we applied a spin angular alignment (smaller than ±3° search angle range and 1° search step) for all particles during the refinement. The symmetry of the MxiG portion (Fig. 1, B and C) was determined after several cycles of focused refinement. The unrolled map of the locally refined MxiG structure (Fig. 1B) was generated by the “unroll” command in UCSF Chimera (45).

To improve the resolution of the WT sorting platform, the six “pods” in each T3SA were first extracted from the original sub-tomograms without binning. From this, we obtained 6 times the “pod” particle number compared with that of the complete WT T3SA. Then 3D focused alignment and classification was used to generate the single “pod” structure.

3D visualization and molecular modeling

UCSF Chimera (45) and ChimeraX (46) were used for the 3D surface rendering of the average structures. The 6-fold symmetrized structure shown in Fig. S1A was first used to generate the 3D surface rendering of the complete T3SA. Then the MxiG component and the “pod” structure were replaced by the local refined structures shown in Fig. S1 (B and C) and Fig. 2 (C–E), respectively, using the “fitmap” function in Chimera or ChimeraX. Atomic models from homologues corresponding to MxiGC (PDB code 3J1W) (47), MxiGP (PDB code 6DUZ), basal body (PDB code 6DV3), rod (PDB code 6DWB) (48), and seatel
focused refined “pod” structure (Fig. 2, C–E) has also been deposited in EM Data Bank (EMD-20561). Software used for cryo-ET analysis in the present work and corresponding references is listed in Table S1.


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