Architecture of the major component of the type III secretion system export apparatus

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Type III secretion systems (T3SSs) are bacterial membrane-embedded nanomachines designed to export specifically targeted proteins from the bacterial cytoplasm. Secretion through T3SS is governed by a subset of inner membrane proteins termed the ‘export apparatus’. We show that a key member of the Shigella flexneri export apparatus, MxiA, assembles into a ring essential for secretion in vivo. The ring-forming interfaces are well-conserved in both nonflagellar and flagellar homologs, implying that the ring is an evolutionarily conserved feature in these systems. Electron cryo-tomography revealed a T3SS-associated cytoplasmic torus of size and shape corresponding to those of the MxiA ring aligned to the secretion channel located between the secretion pore and the ATPase complex. This defines the molecular architecture of the dominant component of the export apparatus and allows us to propose a model for the molecular mechanisms controlling secretion.

RESULTS
MxiA\textsubscript{C} assembles into a homo-nonameric ring
We determined the crystal structure of the ~44 kDa C-terminal cytoplasmic domain of MxiA (residues 318–686, named MxiA\textsubscript{C}; Fig. 1a and Table 1; PDB ID 4a5p), the FlhA homolog from S. flexneri\textsuperscript{21}, a pathogen expressing the T3SS to promote colonic invasion and cell-to-cell spread in the host intestinal epithelium\textsuperscript{22}. As expected, the MxiA\textsubscript{C} monomer had the same fold as its homologs\textsuperscript{15,17–20}. However, to date, a lack of structural details of the supramolecular architecture of the export apparatus has represented one of the major obstacles to deriving a mechanistic model for its activity. We therefore investigated the architecture of the major component of the S. flexneri export apparatus, MxiA.

Fig. 1a

Supplementary Fig. 1

Table 1

Fig. 1b

Supplementary Fig. 2

Fig. 1c

Supplementary Fig. 3

The FlhA homologs from flagellar and pathogenic T3SS\textsuperscript{15,17–20} have demonstrated a conserved fold consisting of four subdomains\textsuperscript{17,20}. However, to date, a lack of structural details of the supramolecular architecture of the export apparatus has represented one of the major obstacles to deriving a mechanistic model for its activity. We therefore investigated the architecture of the major component of the S. flexneri export apparatus, MxiA.

Type III secretion systems (T3SSs) may be broadly divided into two classes, nonflagellar or pathogenic T3SSs, which promote bacterial pathogenesis by secretion of effector proteins directly into host cells\textsuperscript{1–3}, and flagellar T3SSs, which promote bacterial motility\textsuperscript{4}. Both types of T3SSs are evolutionarily closely related, with homologs for most key flagellar T3SS components identified in the pathogenic T3SS and vice versa, and with these homologous pairs exhibiting high structural conservation\textsuperscript{4}. Thus, the overall architecture of the T3SS is generally conserved throughout speciation across both pathogenic secretion and mobility T3SSs.

T3SSs span both bacterial membranes\textsuperscript{5} with a ‘basal body’ from which a needle or a flagellum extends. Secretion through the system is tightly regulated\textsuperscript{6} and depends on a poorly understood conserved inner membrane proteins (Supplementary Table 1) termed the ‘export apparatus’\textsuperscript{7–10} aided by a cytoplasmic ATPase complex\textsuperscript{11}. Although recent work has provided some knowledge of the order of assembly of the components of the export apparatus\textsuperscript{7–9,12}, little is known about its molecular architecture or the molecular mechanisms underlying the control of secretion. The largest of the export apparatus proteins is a member of the FlhA superfamily; polytopic transmembrane proteins with eight predicted transmembrane helices followed by a large cytosolic domain. FlhA homologs have been localized together at the base of the both flagellar and nonflagellar T3SSs\textsuperscript{7,8}, probably in multiple copies\textsuperscript{7–9}, and have been shown to interact with members of the FlhB superfamily\textsuperscript{13,14}, with secretion substrates in complex with their chaperones\textsuperscript{15,16} and with the conserved ATPase and its regulators\textsuperscript{11}. Recent atomic structures of the cytosolic domains of FlhA homologs from flagellar and pathogenic T3SS\textsuperscript{15,17–20} have demonstrated a conserved fold consisting of four subdomains\textsuperscript{17,20}. However, to date, a lack of structural details of the supramolecular architecture of the export apparatus has represented one of the major obstacles to deriving a mechanistic model for its activity. We therefore investigated the architecture of the major component of the S. flexneri export apparatus, MxiA.

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A MxiA ring is required for T3SS secretion in vivo

To test the functional importance of the MxiA C ring, we mutated large residues involved in the subunit interface. In addition to the large surface area buried (~2,000 Å²), the interface is stabilized by five intersubunit salt bridges: Lys504–Asp516, Glu502–Lys548, Glu496–Arg577 and Glu532–Arg560 connect neighboring third subdomains (SD3), and Arg545–Glu418 connects SD3 and SD1 (Fig. 2a and Supplementary Fig. 4). We therefore created mutants, including a variant called MxiACM5 in which all the intermolecular salt bridges are abolished (E502A, K504A, R545A, R560A and R577A). We purified MxiACM5, which produced a circular dichroism spectrum comparable to that of the wild-type protein (Supplementary Fig. 5a), demonstrating that these mutations do not perturb the native folding of the protein. Surface plasmon resonance (SPR) and size-exclusion chromatography coupled to MALD (SEC-MALS) confirmed that MxiAC formed concentration-dependent oligomers in solution (Supplementary Fig. 5b,d). However, SPR and SEC-MALS analyses of MxiACM5 revealed that in solution this variant could not associate productively under conditions identical to those used to assay the wild-type protein (Supplementary Fig. 5c,e). Moreover, purification of wild-type MxiA-Myc–His and full-length, Myc–His-tagged MxiA incorporating the same ring-disrupting mutations as in the MxiACM5 mutant (M5) in a mxiA− S. flexneri background also demonstrated that the wild-type protein could be isolated in an SDS-resistant oligomeric state (Supplementary Fig. 5h) but the M5 variant could not.

We then assayed the effects of MxiA ring-disrupting mutations in vivo by complementing a mxiA− strain with either wild-type or mutant forms of the full-length protein. As previously reported21, the mxiA− strain is secretion-incompetent, but complementation with the wild-type MxiA restored secretion (Fig. 2b). Complementation with the M5 mutant, several triple and quadruple mutants or the R545A mutant did not restore secretion (Fig. 2b). Therefore, mutation of residues predicted on the basis of the crystal structure to disrupt ring formation, led to in vivo secretion deficiencies, supporting the hypothesis that assembly of this MxiA ring is important for biological activity.

Alteration of the MxiA inner pore lining affects secretion

To further probe function, we constructed additional mutations designed to probe the external and internal faces of the ring without disrupting assembly. Earlier work on the MxiA homolog FlhA suggested a role for SD2 (now seen to be arrayed on the external surface and cartoon top and lateral views, (a) Representation of MxiAC ring structure as surface and cartoon top and lateral views, colored by chain. (b) Boundaries of MxiAC subdomains: SD1 (residues 356–428 and 478–493) in red; SD2 (residues 429–477) in yellow; SD3 residues 494–583 in blue; and SD4 residues 584–686 in cyan. The inner ring surface is indicated as a dashed line. (c) Immunoblotting of MxiAC by anti-HisHRP reveals oligomers crosslinked using dimethyl 3,3′-dithiobispropionimidate-2 HCl (DTBP).
of secretion substrates through the center of the MxiA ring is a crucial early step in the secretory pathway.

The ring is conserved in all T3SSs
Homo-nonmeric symmetry is rare and such an assembly has not previously been proposed for the FlhA family. Mapping amino acid conservation onto the monomer (Fig. 4 and Supplementary Fig. 6) revealed that the oligomerizing surfaces are the most highly conserved regions of the structure among both flagellar and non-flagellar homologs. It is also of note that the crystal packing seen in the Salmonella enterica and Bacillus subtilis FlhA structures, both from flagellar T3SSs, depend on very similar subunit interactions (Fig. 4). This conservation of residues in the interface and independent observation of conserved subunit interactions across both flagellar and nonflagellar FlhA family members suggests that they will all assemble into nonameric rings in vivo. We therefore investigated the cross-species conservation of the MxiA nonamer in vivo.

Location of the MxiA ring in vivo
FlhA family members have been shown to associate with the T3SS in fluorescence microscopy studies and have been purified together with T3SSs using gentle extraction methods or in pull-down experiments (Supplementary Table 2). However, the supramolecular architecture and precise location of the FlhA with respect to the T3SS has been ambiguous because of the lack of density in the cytoplasmic regions of current EM reconstructions. To date, the only in vivo imaging of T3SSs is from flagellar systems because the additional components required for torque generation and greater size of the flagellum render these tractable to in situ electron cryo-tomography methods. Imaging of flagellar T3SS from a variety of

Figure 2 In vitro and in vivo analysis of the MxiA ring. (a) Cartoon of the interface between monomers, showing intermolecular salt-bridging residues with their side chains represented as spheres. (b) Immunodetection by western blots of IpaBC in bacterial supernatants (top) and pellet (bottom) for complemented mxiA– Shigella strains upon stimulation with Congo Red. Complementation with M5, R545A, several triple and quadruple mutants did not restore secretion, despite normal expression of secretion substrates in all complemented strains (bottom); expression of all MxiA variants was comparable to that of the wild type (WT), with the only exception of the mutant E502A K504A R560A (anti-His, pellet).
bacterial species revealed a toroidal density at a consistent distance below the cytoplasmic membrane\textsuperscript{32,33} that has been proposed to contain the cytoplasmic domains of FlhA and FlhB. As the dimensions of the torus perfectly match those of the MxiA C ring (Fig. 5a), we investigated whether the torus represents an \textit{in vivo} visualization of FlhA as a nonameric ring. As deletion of the MxiA or FlhA cytoplasmic domains abolished secretion and hence assembly of the outer-membrane and extracellular part of the apparatus (increasing the difficulties of imaging the assembly) we worked in \textit{Campylobacter jejuni} where the flagellar T3SS localizes to a cellular pole and can therefore be located even in the absence of the outer membrane components and flagellum. Electron cryo-tomography produced macromolecular-resolution (2–6 nm) structures of the wild-type flagellar T3SS and the apparatus with cytoplasmic domain truncations of FlhA (ΔFlhAc) or FlhB (ΔFlhBc) (Fig. 5b). As predicted, both the ΔFlhAc and ΔFlhBc reconstructions lacked all components beyond the inner membrane. Comparing wild-type, ΔFlhAc and ΔFlhBc strains revealed that, although the cytoplasmic structures were largely conserved, the toroidal density previously inferred to be a FlhA–FlhB complex\textsuperscript{32,33} was absent in the ΔFlhAc strain but present in both the wild-type and ΔFlhBc strains. In combination with the match between the size and shape of the torus and the MxiA C ring, these mutant tomograms strongly support interpretation of this torus as a FlhA C ring. The position of this ring ~60 Å below the inner membrane is entirely compatible with the length of the linker (45 residues) between the end of the transmembrane helices and the start of FlhA C.

Presence of a density previously ascribed to the ATPase (FliI)\textsuperscript{32} did not change with removal of the FlhA C ring or FlhB C, whereas removal of the ATPase did not affect the density of the torus\textsuperscript{32}. This is consistent with the observation that the ATPase complex and the FlhA homologs associate with the T3SS independently of one another\textsuperscript{7,34}.

It is well known that the T3SS ATPase, a homohexameric enzyme with homology to the α and β subunits of the F\textsubscript{1} ATP synthase, associates with both a central stalk, FliJ (a homolog of the F\textsubscript{1} γ subunit\textsuperscript{33}) and a peripheral stator, FliH (a homolog of the F\textsubscript{1} stator\textsuperscript{36}). Our collection of tomograms\textsuperscript{32} reveal that the distance between the FlhA torus and the ATPase density is consistent between flagellar T3SS from different species, in agreement with the observation that the central stalk protein is relatively invariant in length and of the correct dimensions to bridge the gap. Because the T3SS ATPase is also anchored to the C ring, a cytoplasmic ring structure that varies in diameter across species, by its stator protein\textsuperscript{34}, we investigated the length of the stator
protein in different flagellar T3SSs and discovered that the distance from the ATPase to the C ring is linearly proportional to the length of the stator (Fig. 5c). The increase in size of the C ring (increase in radius of ~1.3 Å per residue of the stator protein) is consistent with the stator adopting an extended helical conformation such as that of the F1-ATPase stator proteins.

Taken together, these data allowed us to build a structural model for the major components of the T3SS export apparatus (Fig. 5a). A ring made of the FlhA family member forms the export gate of T3SSs, with the 72 predicted transmembrane helices forming the pore in the inner membrane in conjunction with the other members of the export apparatus. The large cytoplasmic ring is a key component of the apparatus through which substrates for secretion must pass, thereby controlling access to the secretion pore. The cytoplasmic ATPase complex then docks under the FlhA nonamer via interactions with the C ring.

**DISCUSSION**

Our structure and in vivo work suggest that the *S. flexneri* export apparatus component MxiA and its homologs are biologically active as export rings, and that T3SS secretion most likely proceeds by movement of substrate through the central pore of this assembly. Tomograms of flagellar T3SS lacking the homologous cytoplasmic domain establish that this export ring assembles directly below the basal body in line with the export channel at the center of the apparatus and above the ATPase complex. Furthermore, both the export ring and the ATPase complex assemble independently of each other.

Previous work has demonstrated that the T3SS ATPase complex is structurally homologous to the well-characterized F1-F0 ATP synthase complex, and that secretion by the export apparatus is driven by proton motive force and that the FlhA transmembrane helices are involved in this process.

Using our new architecture to interpret these earlier observations allows us to propose a new model for the way in which FlhA family members interact with the rest of the apparatus to promote secretion of substrates (Fig. 6). In particular, we note that the cytoplasmic export ring pore (Fig. 6a), revealed in our structure of the cytoplasmic domain, is of sufficient size to allow passage of folded or only partially unfolded substrates, in contrast to the pore in the needle or in the flagellum that requires unfolding to the level of isolated helices or extended polypeptide. Work by others has implicated the ATPase complex in recruitment of substrate-chaperone complexes to the export apparatus presumably aided by interactions with other export apparatus components and with the external surface of the export ring in a substrate-specific fashion (this work and refs. 15,16). After separation of the chaperone, we speculate that the largely folded substrate is then admitted to an export cage via the central pore formed by the annular arrangement of the cytoplasmic and transmembrane domains of FlhA homologs and that the proton motive force–driven secretion of the substrate is mediated from this membrane-proximal but relatively isolated location. Our architecture for the major export apparatus component and its positioning with respect to the relatively membrane distant ATPase complex is strongly reminiscent of the architecture of the F1-F0 ATP synthase complex, perhaps implying that there may be greater mechanistic similarities between these systems. Such similarities suggest that one possible mechanism for coupling of ATP hydrolysis at this membrane-distant location to use of proton motive force in secretion is via γ subunit–mediated rotation or induction of conformational change of the export cage. Additional work will be required to test this model and to understand the role of the other export-apparatus components, but we note that there is room for multiple copies of the other key export apparatus components in addition to the nine copies of the FlhA family member, suggested by our structure of the cytoplasmic domain, in the basal body. Differences in the nature of the export-ring pore and system-specific accessory proteins and chaperones will add mechanisms for control of export. We also note that tethering of the ATPase complex to the C ring, which is thought to rotate at least in the flagellar systems, also raises questions about potential additional roles for rotation in driving secretion, which will need to be investigated in future studies.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** The X-ray crystallographic data and coordinates have been deposited in the Protein Data Bank: 4a5p.

**Note.** Supplementary information is available in the online version of the paper.

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**Figure 6** Mechanistic model of T3SS secretion. (a) Slab view of the export cage loaded with substrate (IpA; PDB 2J06; dark gray, left). MxiA model is represented as dark red surface; residues crucial for the function of the export-ring pore (that is, whose mutation dramatically decreases secretion) are highlighted in yellow on the outer surface of the pore. Slab view of the needle with a traversing helix (right). (b) Three-view cartoon of key steps in T3SS secretion: (i) chaperone-effector complex is recruited to the ATPase level; (ii) the ATPase complex strips the chaperone from the exporting substrate, allowing the partially folded substrate to enter the export cage; and (iii) proton motive force mediates secretion of the unfolded substrate through the hollow needle. This simplified diagram does not show the other export apparatus components (Spa24, Spa9, Spa29 and Spa40), although they nucleate the MxiA assembly in the nascent export apparatus or MxiK, which is thought to aid assembly of Spa33.
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**AUTHOR CONTRIBUTIONS**

J.E.D., S.J, and S.M.L initiated the project, which P.A. later joined; S.M.L. and C.M.T. supervised the project. M.E.F. and J.E.D. designed the MxiAC expression vector and did protein expression and stability trials. P.A. performed the large-scale purification, methylation, crosslinking and SPR of MxiAC and its mutants. S.J. performed the MALs experiments. P.A. designed the ‘export apparatus’ co-expression vectors and purified the recombiant complex. P.A. crystallized MxiAC, and optimized crystals for data collection, and S.M.L. soaked and handled crystals for data collection. P.A., P.R. and S.M.L. contributed to the data collection, structure determination and model building. C.M.T. and M.V.-I. designed and performed the complementation and invasion assays in S. flexneri. D.R.H. created the C. jejuni strains, and M.D.B. and G.J.J. designed and performed cryo-EM tomoscopy. P.A., S.J, and S.M.L. analyzed data and wrote the manuscript. All authors read and approved the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Online methods

DNA manipulation, bacterial growth and protein expression conditions. Recombinant expression of MxiA in the *E. coli* C41(DE3) strain was transformed with the plasmid pACYC-6HisMxiA, was obtained with 16 h induction with 1 mM IPTG at 21 °C. For the expression of the export apparatus complex, the *E. coli* L56(DE3) strain was transformed with plasmids pET_MxiA6His and pACYC_Spa24-29ORF, the latter being a polychistronic vector, and induction was carried out at 24 °C for 16 h with 0.1 mM IPTG.

*S. flexneri* mxiA strain was created inserting a double stop codon after the first methionine through homologous recombination with pK303blue plasmid. For complementation assays, *S. flexneri* mxiA strain was transformed with the plasmid expressing the His-tagged full-length MxiA (pBAD_MxiA6His) in the appropriate plasmid by site-directed mutagenesis using the XLQuickChange Kit (Agilent). Constructs were verified by DNA sequencing. Resulting plasmids were transformed into the appropriate host by electroporation or heat-shock method.

*E. coli* strains were grown in LB medium (Invitrogen), Terrific broth (TB; Fisher) or on LB agar. *S. flexneri* strains were propagated in LB broth and LB agar plates with 0.01% Congo Red (CR) in the presence of 0.02% arabinose. Mutations to validate the authenticity and the function of MxiA in *in vitro* and *in vivo* were introduced in the appropriate plasmid by site-directed mutagenesis using the XLQuickChange Kit (Agilent). Constructs were verified by DNA sequencing. Resulting plasmids were transformed into the appropriate host by electroporation or heat-shock method.

Recombinant A complex. Cells expressing both the full length MxiA (1–868), fused to a Myc-His C terminal tag, and Spa24, Spa9 and Spa29 were collected and lysed by homogenization. The clarified lysate was centrifuged at 150,000 g at 1 h at 4 °C to pellet the membrane fraction, which was then solubilized in PBS supplemented with 1% DDM at −15 mg ml−1 for 1 h at 4 °C. After centrifugation at 150,000g for 1 h, the detergent-solubilized complex was purified by IMAC and then dialyzed overnight into PBS with 0.03% DDM.

Native PAGE was performed using Biocad 4–20% Mini-PROTEAN TGX pre-cast gels according to manufacturer instructions at 150 V for 100 min. The gel was then stained for a few minutes in 0.1% Ponceau S solution, and the band of interest was cropped with a sterile blade. The band was then incubated for 30 min in 2× Laemmli loading buffer supplemented with 50 mM DTT, subsequently cast gels according to manufacturer instructions at 150 V for 100 min. The gel was then stained with Coomassie-stained. An equivalent gel was blotted onto a PVDF membrane and the immunodetection was performed using the Qiagen Penta-His membrane and the immunodetection was performed using the Qagen Penta-His HRP Conjugate kit. The identity of the MxiA band was also confirmed by trypsin digest–mass spectrometry analysis (data not shown).

Absolute molar mass was determined using MALD in batch mode by injecting 120 µl of sample at 0.6 mg ml−1 through a system equilibrated in PBS with 0.03% DDM and coupled to multangle light scattering and refractive index detectors (Wyatt Technology). Data analyses were carried out as before.

Electron cryo-tomography. *C. jejuni* strain SNJ833 was grown as images as previously described for *C. jejuni* strain SNJ833. In vitro characterization of the multimeric assembly. Absolute molar mass and mass distribution of purified MxiA was determined using SEC-MALS by injecting through a Superdex 200 10/300 GL or a Superose 6 10/300 GL column equilibrated in a TBS buffer (20 mM Tris–HCl pH 8.0 and 150 mM NaCl) followed in-line by a Dawn Heleos-II light scattering detector (Wyatt Technologies) and an OptLab-Rex refractive index monitor (Wyatt Technologies). Molecular mass calculations were performed using ASTRA 5.3.4.14 (Wyatt Technologies) assuming a refractive index increment (dn/dc) value of 0.186 ml g−1.

MxiA was chemically crosslinked using dimethyl 3,3′-dithiobispropionimi- date-2 HCl (DTBP; Thermo Fisher Scientific). Briefly, MxiA was crosslinked with 10-fold molar excess of DTBP at room temperature for 1 h and then the reaction was quenched adding an excess of Tris–HCl, pH 6.8. Samples treated with and without DTBP were separated by 8% SDS-PAGE and then subjected to western blot using an anti-His HRP–conjugated antibody (Qiagen) for the immunodetection.

Mass spectrometry analyses were performed at the Central Proteomic Facility of Dunn School of Pathology. Automated data analyses were performed using Mascot as search engine in the local proteomic data analysis pipeline.
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