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Received 16 Jan 2015 | Accepted 7 Apr 2015 | Published 21 May 2015

DOI: 10.1038/ncomms8125

OPEN

A common assembly module in injectisome and flagellar type III secretion sorting platforms

Ryan Q. Notti^{1,2}, Shibani Bhattacharya³, Mirjana Lilic¹ & C. Erec Stebbins¹

Translocating proteins across the double membrane of Gram-negative bacteria, type III secretion systems (T3SS) occur in two evolutionarily related forms: injectisomes, delivering virulence factors into host cells, and the flagellar system, secreting the polymeric filament used for motility. While both systems share related elements of a cytoplasmic sorting platform that facilitates the hierarchical secretion of protein substrates, its assembly and regulation remain unclear. Here we describe a module mediating the assembly of the sorting platform in both secretion systems, and elucidate the structural basis for segregation of homologous components among these divergent T3SS subtypes sharing a common cytoplasmic milieu. These results provide a foundation for the subtype-specific assembly of T3SS sorting platforms and will support further mechanistic analysis and anti-virulence drug design.

¹Laboratory of Structural Microbiology, Rockefeller University, 1230 York Avenue, New York, New York 10065, USA. ²Tri-Institutional Medical Scientist Training Program, Weill Cornell Medical College, 1300 York Avenue, New York, New York 10021, USA. ³New York Structural Biology Center, 89 Convent Avenue, New York, New York 10027, USA. Correspondence and requests for materials should be addressed to C.E.S. (email: stebbins@rockefeller.edu).

Type III secretion systems (T3SS) allow the transport of protein substrates directly across the double membrane of Gram-negative bacteria. There are two evolutionarily related, yet functionally distinct subtypes of T3SS: ‘injectisomes’, which deliver effector proteins into the cytoplasm of eukaryotic host cells¹, and the flagellar apparatus, which secretes the polymeric filament used for motility². Despite their functional divergence, injectisomes and the flagella share a common core of homologous gene products and possess ultrastructural similarities³. For example, both systems share related elements of a ‘sorting platform’ that facilitates the hierarchical secretion of protein substrates⁴.

Proteomic analyses have identified the major components of the sorting platform for the *Salmonella typhimurium* SPI-1 injectisome: the AAA + ATPase InvC, its regulator OrgB and the proteins SpaO and OrgA⁴. While SpaO has been shown to be necessary for formation of the sorting platform⁴, little is known about its molecular structure. In *Yersinia*, the SpaO homologue is expressed as a full-length protein as well as a carboxy-terminal fragment translated from an internal translation start site⁵; this carboxy-terminal fragment dimerizes and can interact with the full-length protein. The crystal structure of the *Yersinia* carboxy-terminal dimer is similar to that of its *Pseudomonas*⁶ and flagellar⁷ homologues, together characterizing a structural class known as the surface presentation of antigens (SPOA) domain. Whether other domains within SpaO possess a similar structure, and how these structures correlate with function remains unknown.

In the flagellar apparatus, the SpaO homologues FliM and FliN form a robust, stable ring (the ‘C-ring’) at the cytoplasmic face of the basal body⁸. Electron microscopic analyses have similarly localized the SpaO homologue to the cytoplasmic face of the *Shigella* injectisome⁹, and recent cryoelectron tomographic studies in the same organism identified SpaO homologue-dependent ‘pods’ of density beneath the injectisome¹⁰.

In contrast to the flagellar C-ring, this sub-injectisome structure is less robust¹⁰, and fluorescence microscopic analysis of the *Yersinia* SpaO homologue show that there is dynamic exchange between cytoplasmic- and injectisome-associated forms¹¹. How SpaO and its homologues interact with other elements of the T3SS has yet to be shown at high resolution, and how homologous flagellar and injectisome components are properly segregated to their cognate secretion systems remains an open question.

Here we show that a novel, heterotypic interaction between SPOA domains serves as a scaffold for sorting platform assembly in both injectisome and flagellar T3SS. Solution nuclear magnetic resonance (NMR) data support the crystallographic model, and structure-guided mutagenesis shows that this interaction is necessary for formation of the SpaO–OrgB–InvC complex, the proper localization of SpaO to the bacterial inner membrane and T3SS function. Structures of the flagellar SpaO–OrgB homologues FliM, FliN and FliH reveal a mechanism for the proper segregation of homologous sorting platform components among T3SS subtypes sharing a common cytoplasmic milieu. Together, these structures define a common module utilized in sorting platform assembly and provide insight into the subtype-specific assembly of T3SS.

Results

SpaO contains two bona fide SPOA domains. To dissect the structural basis for the sorting platform assembly, we determined the structures of individual domains of *S. typhimurium* SpaO and then characterized their interactions with other sorting platform components. Preliminary bioinformatic analyses suggested the presence of two putative SPOA domains in the carboxy-terminal half of SpaO, which we denote SPOA1 and SPOA2 (Fig. 1a). We first determined the structure of the SPOA2 homodimer to 1.35 Å resolution (Fig. 1b,c; Table 1; Supplementary Fig. 1). The SPOA2 homodimer structure is architecturally similar to its

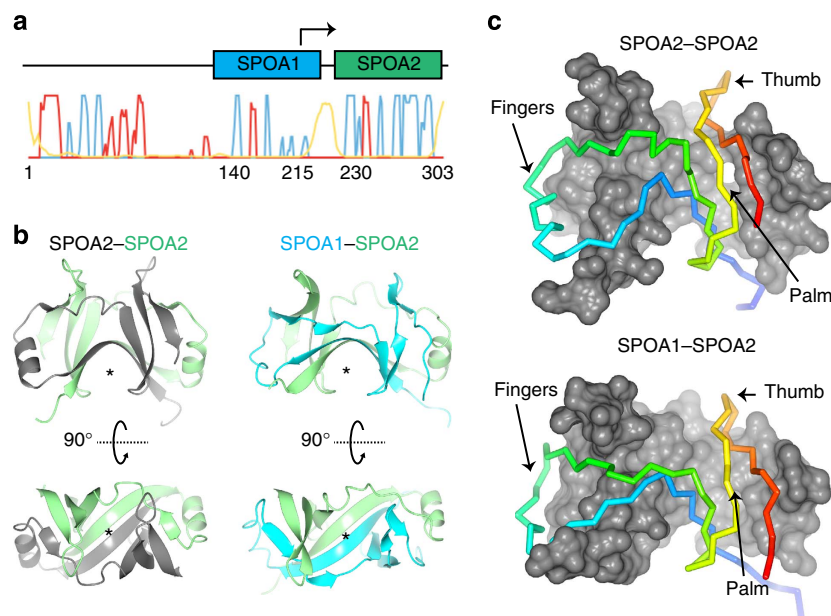


Figure 1 | Homotypic and heterotypic SPOA interactions. (a) Bioinformatic analysis of SpaO. PSIPRED secondary-structure predictions and sequence homology suggest the presence of two putative SPOA domains in SpaO. Probability of helical character is plotted in red, strand in blue and disorder in yellow. The arrow at codon 203 represents a predicted Val_{GTE} internal translation start site, as has been shown for YscQ Met218 in *Yersinia pseudotuberculosis*⁵. (b,c) Comparison of homotypic SPOA2–SPOA2 and heterotypic SPOA1–SPOA2 structures from SpaO. (b) Ribbon diagrams show the similar organization of secondary-structural elements in both SPOAs. Asterisks denote the antiparallel beta-sheet ‘floor’, (c) Amino (blue) to carboxy (red) C α traces of SPOA2 (top) and SPOA1 (bottom) reveal a similar topology in interaction with SPOA2 (grey surface representation, top and bottom).

Table 1 | Data collection and refinement statistics for injectisome structures.

	SpaO(232–297, SeMet)	SpaO(145–213, SeMet) + SpaO(232–297, SeMet)	SpaO(145–213) + SpaO(232–297)	SpaO(145–213) + SpaO(232–297) + OrgB(1–30):: lysozyme	SpaO(145–213, SeMet) + SpaO(232–297, SeMet) + OrgB(1–30)::lysozyme
<i>Data collection</i>					
Space group	P 2 ₁	P 4 ₁ 2 ₁ 2	P 4 ₁ 2 ₁ 2	P 2 ₁	P 2 ₁
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	35, 41.27, 48	66.38, 66.38, 95.21	65.76, 65.76, 95.65	62.092, 89.07, 62.092	62.88, 88.5, 63.32
α , β , γ (°)	90, 103.92, 90	90, 90, 90	90, 90, 90	90, 114.94, 90	90, 116.07, 90
Resolution (Å)	31.26–1.35 (1.37–1.35)	46.94–3.00 (3.18–3.00)	38.68–2.9 (3.08–2.9)	47.59–2.0 (2.05–2.0)	45.8–2.35 (2.43–2.35)
<i>R</i> _{merge}	0.146 (1.281)	0.221 (1.463)	0.166 (1.447)	0.102 (0.530)	0.088 (0.617)
<i>I</i> / σ <i>I</i>	8.6 (2.1)	11.8 (3.1)	14.4 (2.7)	10.5 (3.2)	12.8 (2.6)
CC _{1/2}	0.994 (0.750)	0.989 (0.839)	0.996 (0.856)	0.994 (0.828)	0.997 (0.816)
Completeness (%)	99.7 (100)	100 (100)	99.3 (99.3)	99.5 (99.8)	99.0 (99.6)
Redundancy	7.0 (7.1)	25.6 (27.2)	24.6 (26.2)	5.1 (5.2)	6.6 (6.6)
<i>Refinement</i>					
No. of reflections	29,246		4,964	41,183	25,740
<i>R</i> _{work} / <i>R</i> _{free}	0.1724/0.2053		0.2085/0.2795	0.1571/0.2096	0.1984/0.2618
No. of atoms	1,286		1,024	5,769	4,940
Protein	1,062		1,023	5,112	4,818
Ligand/ion	2		1	0	0
Water	222		0	657	122
<i>B</i> factors					
Protein	14.70		74.20	33.10	46.90
Ligand/ion	14.20		105.00		
Water	32.10			39.60	45.60
r.m.s.d.					
Bond lengths (Å)	0.007		0.010	0.008	0.011
Bond angles (°)	1.09		1.33	1.16	1.46

SeMet, selenomethionine.

homologues^{5,6}: like two left hands grasping one another, an antiparallel beta-sheet ‘palm’ of each protomer is grasped by the ‘fingers’ of the other, with a ‘thumb’ protruding from the top of the palm and strands from each protomer forming an antiparallel beta sheet on the ‘floor’ of the assembly (Fig. 1b,c). The *Salmonella* SPOA2 homodimer superposes on its *Yersinia* and *Pseudomonas* homologues with 2.24 and 3.05 Å root mean squared deviation (r.m.s.d.), respectively (Supplementary Fig. 1b).

While SPOA1 alone was insoluble, constructs containing both SPOA1 and SPOA2 (residues 140–297) were stable and soluble. SpaO(140–297) was analysed by solution NMR (Supplementary Fig. 2a), and chemical shift deviation (CSD) analysis of backbone amide resonances suggested a secondary-structure pattern similar to that predicted by bioinformatic analyses: two SPOA domains connected by a flexible linker (Supplementary Fig. 2b). We hypothesized that SPOA2 interacts with and stabilizes SPOA1; consistent with this hypothesis, a SPOA1 construct (145–213) could be co-refolded with SPOA2. This complex crystallized, and its structure was determined to 2.9 Å resolution (Fig. 1b,c; Table 1; Supplementary Fig. 3). SPOA1 and SPOA2 form a distinct, heterotypic SPOA–SPOA interaction with an overall topology similar to that of the SPOA2 homodimer. The SPOA1 backbone follows that of the prototypical SPOA fold, retaining the antiparallel beta-sheet floor and fingers-to-palm architecture (Fig. 1b,c). In both SPOA1,2 and the SPOA2 homodimer, interacting protomers each bury about 1,800 Å² against their binding partner. SPOA1,2 and the SPOA2 homodimer superpose with 2.47 Å r.m.s.d. (Supplementary Fig. 3b), and the conformation of SPOA2 in association with SPOA1 is grossly similar to that seen in the homodimer, superposing with an r.m.s.d. of 1.67 Å (Supplementary Fig. 3c).

Further supporting the hypothesis that SPOA1 and SPOA2 interact in solution, a *post hoc* analysis of the three dimensional ¹⁵N-edited nuclear Overhauser enhanced spectroscopy-(3D NOESY-HSQC) for SpaO(140–297) revealed long-range amide proton correlations between SPOA1 and SPOA2 (Supplementary Fig. 4). Given the <20 residue linker connecting SPOA1 and SPOA2, they would experience a low millimolar-range relative concentration and would likely interact in an intramolecular fashion (Fig. 2a). However, at high local SpaO concentrations (for example, in association with the T3SS), intermolecular heterotypic SPOA interactions might explain the apparent oligomeric nature of the sorting platform (Fig. 2a). Indeed, a similar model of intermolecular domain swapping was recently suggested for the ring-forming injectisome protein PrgK¹².

Hypothetical SpaO oligomerization driven by intermolecular heterotypic SPOA interactions would be dependent on the covalent linkage of SPOA1 and SPOA2. Thus, we tested whether genomic deletion of the SpaO amino-terminal domain and SPOA1 can be complemented in trans, as assayed by *Salmonella* T3SS function *in vitro*. When grown under T3SS-stimulating conditions, the culture supernatant of *S. typhimurium* has a stereotyped protein composition, consisting of both flagellar and injectisome secretion substrates (Fig. 2b, secreted proteins are annotated as per Mizusaki *et al.*¹³). Deletion of *spaO* results specifically in the loss of injectisome-dependent secretory products from the culture supernatant, and deletion of *spaO* codons 1–203 phenocopies *spaO* deletion, indicating that the SpaO amino-terminal domain(s) and/or SPOA1 are necessary for T3SS function (Fig. 2c). Because SpaO(1–219) is able to complement the deletion of *spaO* codons 1–203 (Fig. 2c, red asterisks), the covalent linkage of SPOA1 and SPOA2 is not

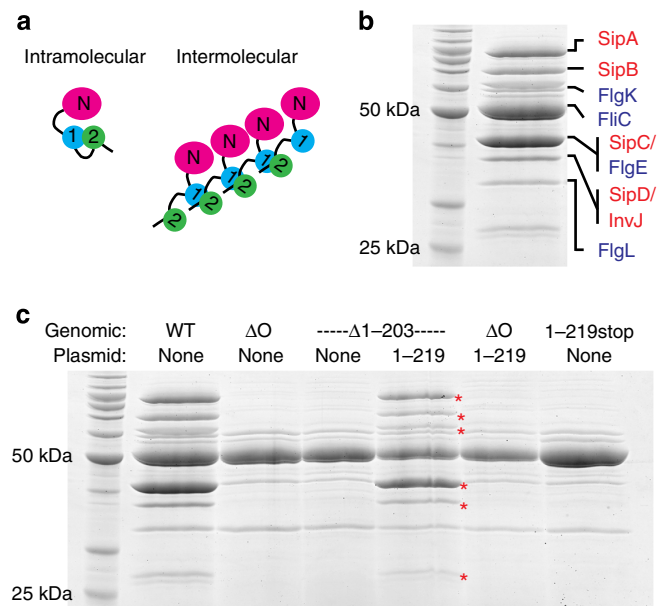


Figure 2 | Intermolecular SPOA1-SPOA2 interactions are not necessary for T3SS function. (a) Schematic models for putative intra- and intermolecular SPOA1-SPOA2 interactions and their implications for the SpaO oligomerization state. The numbers 1 and 2 indicate the SpaO SPOA1 and SPOA2, respectively, and N indicates the SpaO amino-terminal domain(s). (b) Coomassie-stained polyacrylamide gel electrophoresis (PAGE) of *S. typhimurium* culture supernatants grown under T3SS-stimulating conditions (0.3 M NaCl, strain SB1741). Bands previously identified by Mizusaki *et al.*¹³ are noted and colour coded by the T3SS subtype—injectisome in red and flagellar in blue. (c) Coomassie-stained PAGE of *S. typhimurium* culture supernatants grown under T3SS-stimulating conditions. Red asterisks indicate injectisome-specific secretion substrates. WT, wild type; ΔO , deletion of *spaO*; $\Delta 1-203$, deletion of *spaO* codons 1-203; 1-219, complementation with SpaO(1-219); 1-219stop, insertion of two stop codons following *spaO* codon 219. SpaO was 3 × FLAG tagged at its amino terminus in each *S. typhimurium* strain (except ΔO) and complementation construct. Data shown are representative of three experiments.

necessary for T3SS function. Thus, if intermolecular heterotypic SPOA interactions do occur *in vivo*, they are not explicitly necessary for secretion. It should be noted that SpaO(1-219) does not complement a full genomic deletion of *spaO*, demonstrating that SPOA2 is also necessary for T3SS function (Fig. 2c). Similarly, insertion of a double stop codon after *spaO* codon 219 abrogates T3SS (Fig. 2c).

SpaO SPOA1,2 is a scaffold for interaction with OrgB-InvC.

Double-hexahistidine-tagged SpaO is able to co-affinity purify the sorting platform components OrgB and InvC when co-expressed in *Escherichia coli* (Fig. 3a). Formation of the SpaO-InvC-OrgB ternary complex is OrgB dependent, as SpaO alone is insufficient to co-affinity purify InvC (Fig. 3a). We hypothesized that SPOA1,2 might serve as a scaffold for the interaction of SpaO with OrgB-InvC. Indeed, SPOA1,2 is sufficient to co-affinity purify OrgB-InvC (Fig. 3a). This construct contains a Val203_{GTT}-to-Val203_{GTT} mutation to prevent the duplicitous translation of SPOA2 from its cryptic internal translation start site, demonstrating that the SPOA2 homodimer is dispensable for SpaO-InvC-OrgB complex formation.

OrgB and its homologues are predicted to share a common amino-terminal organization: a disordered region followed by a coiled coil. In the flagellar system, the unstructured region at the

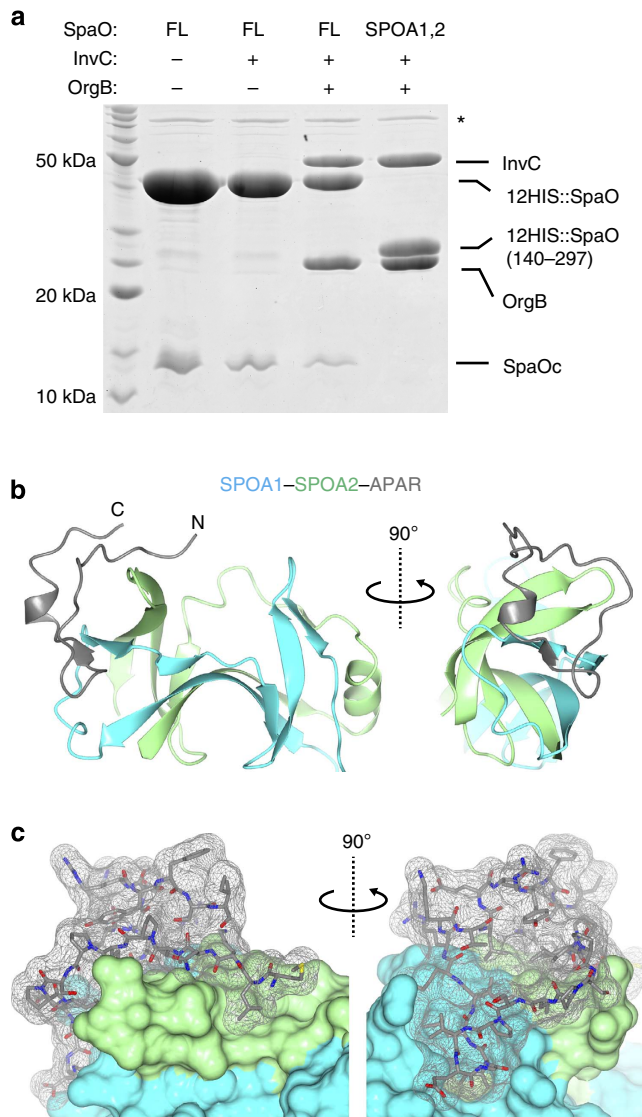


Figure 3 | Architecture of the SPOA1,2-APAR interaction.

(a) Coomassie-stained gel of protein elution from NiNTA resin shows that double-hexahistidine (12HIS)-tagged SpaO ('FL', full length) and SpaO(140-297, Val203_{GTT}) are each sufficient to co-affinity purify InvC-OrgB when co-expressed in *Escherichia coli*. Note that OrgB is necessary for ternary complex formation. Asterisk denotes nonspecific co-purifying *E. coli* proteins, likely chaperones. SpaOc indicates the cryptically expressed SPOA2-containing carboxy-terminal fragment. Data shown are representative of three experiments. (b) Ribbon diagram of the SpaO-InvC-OrgB crystal structure. For simplicity, the T4 lysozyme crystallization chaperone has been omitted and only one of the two constituent complexes from the crystallographic asymmetric unit is shown. The amino and carboxy termini of the OrgB APAR are denoted as 'N' and 'C', respectively. (c) Surface representation of the complex in b. The OrgB APAR (grey mesh) contacts both SpaO SPOA1 (cyan) and SPOA2 (green).

amino terminus of the OrgB homologue FliH is necessary for its interaction with the SpaO homologues FliM and FliN¹⁴. We solubilized the pre-coiled-coil region of OrgB (residues 1-30) by genetic fusion to T4 lysozyme and found that it bound to SpaO SPOA1,2. Herein, we will refer to the SPOA1,2-binding region at the amino terminus of OrgB and its homologues as the adaptor peptide of the ATPase regulator (APAR).

The SPOA1,2-OrgB(APAR)::lysozyme complex was crystallized and its structure solved to 2.0 Å resolution (Fig. 3b; Table 1;

Supplementary Figs 5 and 6). The OrgB APAR forms a lariat-like structure, contacting the thumb of SPOA2 and fingers of SPOA1 (Fig. 3b,c). OrgB makes substantial contact with both SPOA1 and SPOA2 of SpaO, burying 570 \AA^2 against SPOA1 and 470 \AA^2 against SPOA2. In the APAR-bound structure, there is little change in the conformation of SpaO (Supplementary Fig. 5d, 1.01 \AA r.m.s.d.).

Independent NMR analyses of SpaO(140–297) bound to OrgB(APAR) in solution are consistent with the interface defined in the crystal (Fig. 4). Compared with apo-SpaO, the largest CSDs of backbone amide resonances in SpaO–OrgB map on the crystal structure to residues involved in the interface, which are highly conserved across both the *Salmonella/Shigella* and *Yersinia/Pseudomonas* clades (Fig. 4; Supplementary Fig. 7). In the crystal, these residues form the docking site for OrgB residues Ile17, Leu18 and Ile19 (Fig. 5a). The OrgB surface area buried by these three residues (360 \AA^2) accounts for approximately one-third of the APAR's total buried area. Here, the APAR shows noteworthy sequence homology: immediately following a conserved glycine (Gly16, pseudo-lariat apex) is a string of aliphatic and basic amino acids in each homologue (Fig. 5a).

The SPOA1,2–APAR interaction is necessary for T3SS function.

To test whether the SPOA1,2–APAR interaction *per se* is necessary for T3SS function, we constructed an OrgB triple mutant (I17D,L18D,I19D) to disrupt its interaction with SpaO. As predicted, SpaO failed to co-affinity purify OrgB(I17D,L18D,I19D)–InvC when co-expressed in *E. coli* (Fig. 5b), and the aspartate triple mutation completely abolished T3SS *in vivo* (Fig. 5c). Fluorescence microscopic analyses of the *Yersinia* SpaO homologue have shown it to localize in discrete perimembranous punctae¹¹. Might the SPOA1,2–APAR interaction function to localize SpaO to the bacterial inner membrane? In an otherwise wild-type genomic background, an EGFP::3 × FLAG::SpaO fusion

exhibits punctate, perimembranous localization, consistent with its recruitment to injectisome basal body channels (Fig. 5d). Deletion of *orgB* disrupts proper SpaO localization, producing a more diffuse, cytoplasmic pattern, and the aspartate triple mutation was sufficient to phenocopy the *orgB* deletion mutant (Fig. 5d). Together, these data suggest that the SpaO(SPOA1,2)–APAR assembly is necessary for the proper localization of SpaO to discrete perimembranous puncta, and that this arrangement is required for T3SS function.

A divergent SPOA1,2–APAR assembly in the flagellar T3SS.

The flagellar C-ring is primarily composed of three proteins: FliM, FliN and FliG⁸. The SpaO homologues FliM and FliN are predicted to contain one SPOA domain each, which we designate as SPOA1 and SPOA2, respectively. Paralleling the injectisome, FliN is known to interact with the OrgB homologue FliH¹⁴. The evolutionary relationship between injectisomes and flagella creates a practical conundrum: how are homologous T3SS components segregated to their corresponding secretion systems within a common cytoplasmic milieu? To qualitatively assess the subtype specificity of SPOA–APAR interactions, we co-affinity purified a panel of *Salmonella* SPOA domains with hexahistidine-tagged APAR::lysozyme fusions (Fig. 6). Indeed, the OrgB and FliH APARs robustly co-affinity purify their cognate SPOA1,2 but not that of the other T3SS subtype (Fig. 6, red asterisks). Neither SpaO nor FliM–FliN are pulled down by the APAR from a second pathogenic T3SS found in *S. typhimurium* (SPI-2 SsaK). Consistent with the observation that the OrgB APAR interacts with surfaces on both SPOA1 and SPOA2, the OrgB and FliH APARs more robustly pull down their cognate SPOA1,2 than homodimeric SPOA2 (Fig. 6).

We hypothesized that divergence of the SPOA1,2–APAR assembly architecture contributes to proper component segregation among T3SS subtypes, and sought to structurally

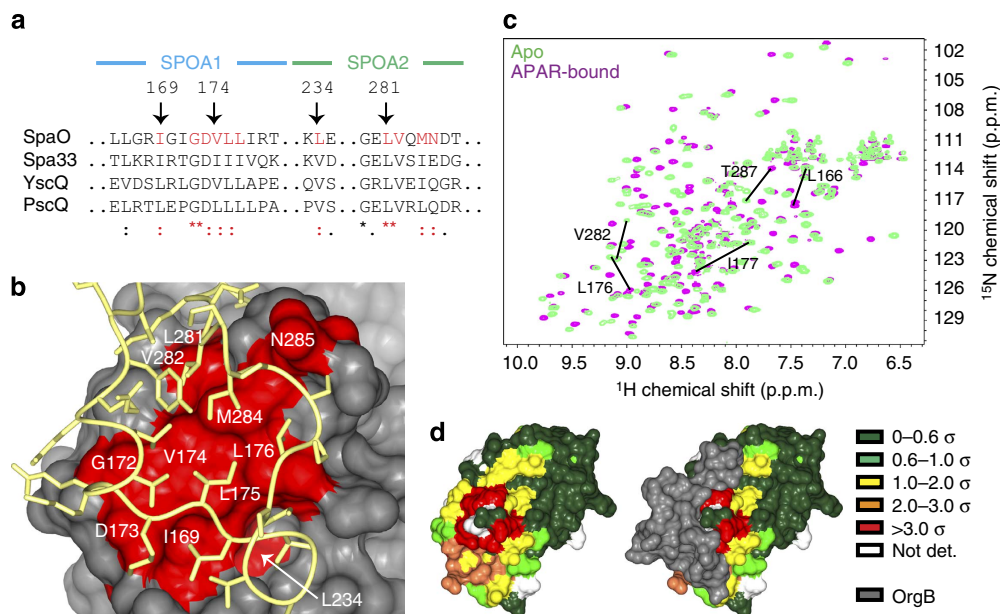


Figure 4 | The APAR-binding site of SpaO. (a) The SpaO residues at the APAR interaction site are highly conserved across homologues in other species. Excerpts of the M-COFFEE alignment of SpaO, *Shigella flexneri* Spa33, *Yersinia enterocolica* YscQ and *Pseudomonas aeruginosa* PscQ are shown with conserved APAR-interacting residues highlighted in red. Symbols beneath the alignment indicate the degree of conservation: asterisks denote full conservation, colons denote strong similarity, and dots denote weak similarity. (b) A surface representation of SpaO with the conserved interfacial residues identified in a are coloured red and the OrgB APAR backbone is yellow. (c) Overlaid ^{15}N -heteronuclear single quantum coherence spectra of apo- (green) and APAR-bound (violet) SpaO(140–297). The five largest peak shifts are noted. (d) The solution interaction data from c are mapped onto the SpaO–OrgB crystal structure. Surface residues are colour coded by the size of their weighted CSD in units of s.d. Residues not assigned an amide resonance in one of the two data sets are left white. The same view of SpaO is shown with and without OrgB (grey surface).

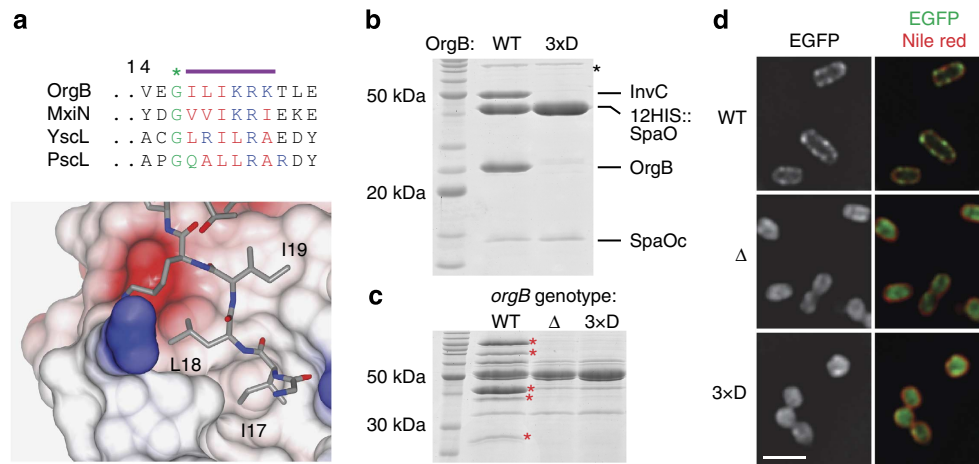


Figure 5 | Structure-guided disruption of the SPOA1,2-APAR interaction. (a) Clustal Omega alignment of the APAR regions of OrgB, *S. flexneri* MxiN, *Y. enterocolica* YscL and *P. aeruginosa* PscL. The conserved pseudo-lariat apex glycine is indicated by a green asterisk and the subsequent patch of aliphatic (red) and basic (blue) amino acids is highlighted with a purple bar. Beneath, the binding site for OrgB(17–19) (grey) is shown as an electrostatic surface. OrgB(1–15) have been removed for clarity. (b) Coomassie-stained polyacrylamide gel electrophoresis of the protein elution from NiNTA resin shows that double-hexahistidine-tagged SpaO can co-affinity purify wild-type InvC–OrgB but not InvC–OrgB(I17D,L18D,I19D) when co-expressed in *E. coli*. 3 × D indicates the OrgB(I17D,L18D,I19D) triple mutant. Asterisk denotes nonspecific co-purifying *E. coli* proteins, likely chaperones. SpaOc indicates the cryptically expressed SPOA2-containing carboxy-terminal fragment. (c) Coomassie-stained culture supernatant from wild-type (WT, strain SB1741), *orgB* deletion (Δ) and *orgB*(I17D,L18D,I19D) (3 × D) *S. typhimurium* shows loss of injectisome substrate (red asterisks) secretion in the mutants, while flagellar secretion remains intact. (d) Widefield microscopic imaging of fixed *S. typhimurium* shows exclusive perimembranous localization of SpaO in the WT background, but cytoplasmic localization in the *orgB* mutants (scale bar, 2 μ m, single z-slices shown). Data shown in b–d are representative of three experiments.

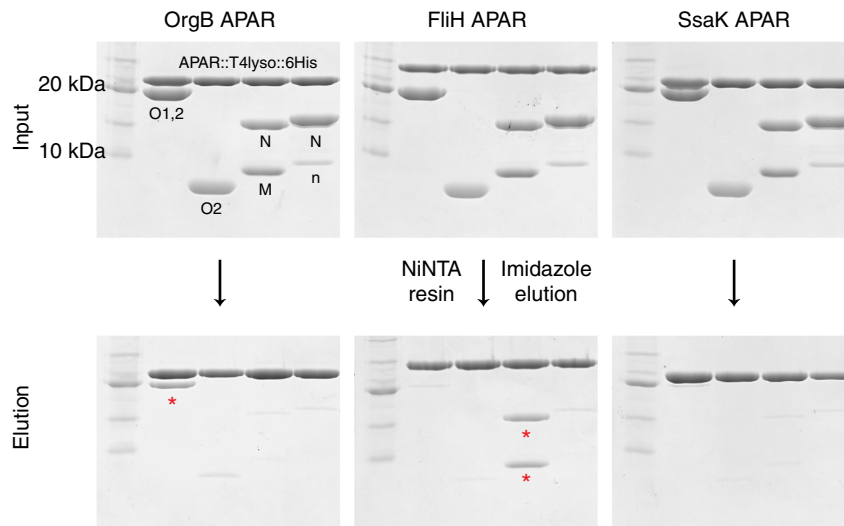


Figure 6 | APARs preferentially interact with their cognate SPOA1,2. Coomassie-stained gels showing the input and imidazole elution for APAR–SPOA co-affinity purification experiments. Red asterisks indicate the cognate SPOA1,2 band(s). T4lyso, T4 lysozyme; O1,2, SpaO(140–297); O2, SpaO(232–297); M, FliM(245–320); N, FliN (1–137); n, co-purifying amino-terminal FliN degradation product. Data shown are representative of three experiments.

characterize the flagellar SPOA–APAR interactions. While complexes of FliM and FliN were stable, they were resistant to crystallization. Interestingly, FliM and FliN can be fused and still support flagellin secretion (Supplementary Fig. 8a) and some swarming motility¹⁵. We crystallized the SPOA of FliM (residues 245–334) fused to FliN(5–137), and its structure was solved to 2.56 Å (Table 2; Supplementary Fig. 8). Architecturally, the FliM(SPOA1)–FliN(SPOA2) interaction is similar to that of SpaO (Supplementary Fig. 8e, 2.28 Å r.m.s.d.), with the exception of additional helices present at the carboxy terminus of each SPOA, as observed in FliN homodimers from *Thermotoga maritima*⁷. The similarity of these structures is consistent with

the SPOA heterotypic interaction being generalizable across T3SS subtypes.

To elucidate the mechanism of FliH-specific assembly with FliM–FliN, we co-crystallized the FliM(SPOA)::FliN fusion with a FliH(1–18)::lysozyme fusion (Fig. 7; Table 2; Supplementary Fig. 9). As with its injectisome counterparts, the FliM–FliN SPOA1,2 did not undergo large conformational changes upon APAR binding (Supplementary Fig. 9c, 1.11 Å r.m.s.d.); however, the binding mode for the FliH APAR is radically different. In contrast to the OrgB pseudo-lariat, the FliH APAR adopts a near-linear conformation along the ‘top’ of FliM–FliN (Fig. 7a). As observed in the SpaO–OrgB assembly, the FliH APAR makes

extensive contact with both SPOA1 and SPOA2 (Fig. 7a), supporting the observation that the FliH APAR interacts more strongly with the FliM–FliN heterodimer than the FliN homodimer (Fig. 6).

The FliH–FliM–FliN assembly is characterized by the burial of several highly conserved hydrophobic FliH side chains. Two

tryptophan side chains form an aromatic clamp, which binds hydrophobic pockets on opposite faces of the FliN thumb (Fig. 7b). These residues are critical for flagellar function¹⁴ and are highly conserved (Fig. 7c). Similarly, the bulky side chain of FliH Leu15 fills a hydrophobic pocket on the thumb of FliM (Supplementary Fig. 10). The binding interfaces for these three residues are formed by both FliM and FliN and are highly conserved across species (Supplementary Fig. 10). This structure presents a conserved model for FliH–FliM–FliN interaction, which is distinct from that of SpaO–OrgB.

Table 2 | Data collection and refinement statistics for flagellar structures.

	FliM(245–334):: FliN(5–137), SeMet	FliM(245–334):: FliN(5–137) + FliH(1–18)::lysozyme
<i>Data collection</i>		
Space group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	75.15, 81.50, 89.96	43.21, 76.37, 119.4
α , β , γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	57.67–2.56 (2.67–2.56)	64.33–2.30 (2.38–2.30)
<i>R</i> _{merge}	0.097 (1.215)	0.070 (0.923)
<i>I</i> / σ <i>I</i>	18.5 (2.7)	20.2 (2.6)
CC _{1/2}	0.999 (0.814)	0.999 (0.811)
Completeness (%)	100 (100)	99.8 (99.8)
Redundancy	13.8 (14.3)	12.9 (12.9)
<i>Refinement</i>		
No. of reflections	18,372	18,174
<i>R</i> _{work} / <i>R</i> _{free}	0.2175/0.2593	0.1967/0.2620
No. of atoms	2,633	2,739
Protein	2,605	2,668
Ligand/ion	5	0
Water	23	71
<i>B</i> factors		
Protein	68.30	69.70
Ligand/ion	73.40	
Water	64.50	65.00
r.m.s.d.		
Bond lengths (Å)	0.010	0.009
Bond angles (°)	1.31	1.15

SeMet, selenomethionine.

Discussion

We present here a series of structures that yield critical mechanistic insights into T3SS sorting platform assembly across multiple species and secretion subtypes. The existence of heterotypic SPOA interactions provides a structural explanation for the observed ~1:3 stoichiometry of SPOA1 to SPOA2 in SpaO homologues⁵. While two of these SPOA2 domains could be accounted for by a homodimer interacting with full-length SpaO, the conformation of the third SPOA2 (located in the full-length protein) was unclear. Previous reports had proposed the existence of an alternate autostabilizing conformation for the third SPOA2 (ref. 5). We show here that the third SPOA2 can be stabilized by a SPOA1–SPOA2 interaction.

Similar to SpaO and its injectisome homologues, the ratio of FliM to FliN *in situ* is estimated to be 1:3 (ref. 8). In the context of our FliM–FliN structure, this suggests a model for FliM–FliN interaction similar to that of SpaO. FliM(SPOA1) would engage FliN(SPOA2) in a heterotypic SPOA–SPOA interaction, and additional homodimeric FliN would interact with FliM–FliN in an as of yet undetermined fashion (analogous to the SpaO SPOA2 homodimer interaction with full-length SpaO). However, reports of FliN tetramerization and FliM:FliN ratios between 1:3 and 1:4 suggests that more complicated higher-order structures may be used by the flagellar apparatus⁷. It should also be noted that while previous investigations of the flagellar T3SS have focused on the interaction between FliH and FliN specifically¹⁴, our structures and biochemical data show that the FliH APAR more strongly interacts with the FliM–FliN complex than with FliN alone,

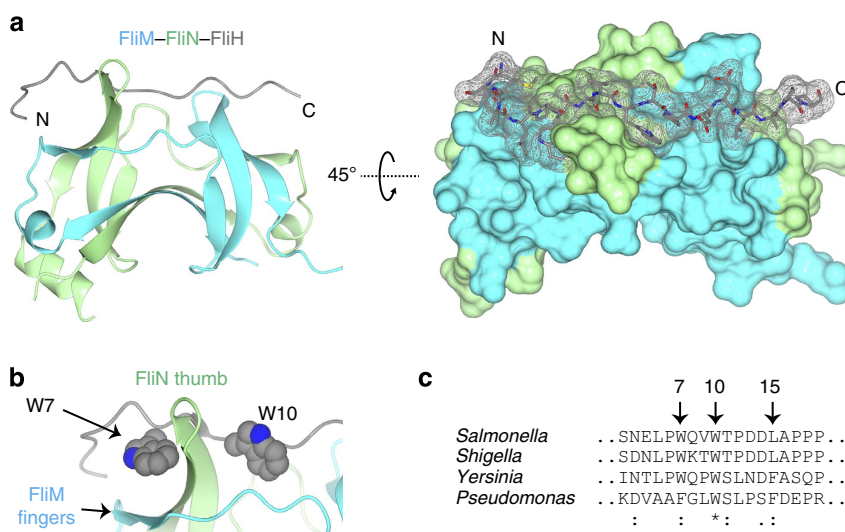


Figure 7 | Structure of the SPOA1,2-APAR interaction in the flagella. (a) Ribbon diagram (left) and surface representation (right) of the FliM–FliN–FliH structure. T4 lysozyme has been omitted. N and C indicate the amino and carboxy termini of the FliH APAR, respectively. (b) A zoomed view of the FliH aromatic clamp, with the side-chain atoms of FliH W7 and W10 represented as spheres. (c) Excerpted M-COFFEE alignment of FliH with its homologues from *S. flexneri*, *Y. enterocolica* and *P. aeruginosa*. Highly conserved residues of interest are noted (*S. typhimurium* numbering). Symbols beneath the alignment indicate the degree of conservation: asterisks denote full conservation, colons denote strong similarity, and dots denote weak similarity.

suggesting that the FliM–FliN complex is the physiologically relevant binding partner for FliH.

Our structures suggest a partial model for the subtype-specific assembly of the T3SS sorting platforms: the heterotypic interaction between SPOA domains within a given T3SS subtype functions as an adaptor for ATPase and its regulator through interaction with the APAR peptide (Fig. 8). However, a number of questions remain regarding the higher-order architecture of the sorting platform *in situ*. We hypothesize that the puncta formed by SpaO *in vivo* represent the high-molecular weight sorting platforms described by Lara-Tejero *et al.*⁴. Diepold *et al.* have quantified the stoichiometry and dynamics of these puncta in *Yersinia*, showing them to possess ~22 copies of the SpaO homologue per punctum and to be in dynamic exchange with the cytoplasm¹¹. In contrast, the recent tomographic reconstruction of *Shigella* injectisomes by Hu *et al.* revealed the presence of only six SpaO homologue-dependent pods of density beneath the injectisome, and their localization was OrgB homologue independent¹⁰. Taken together with our findings, these results suggest that there may be two subpopulations of SpaO *in vivo*: one stably associated with the injectisome, and a second dynamic population in exchange with the cytoplasm, requiring the SPOA1,2–APAR interaction to form high-molecular weight, perimembranous sorting platforms. Recent analyses of FliI ATPase dynamics by Bai *et al.* suggest a similar two-population model, which they hypothesize functions to deliver secretion substrates to the assembling flagella¹⁶.

What might be the mechanism for sorting platform targeting, and how might this factor into T3SS machine function? Perhaps APAR binding to the SPOA1,2 scaffold induces conformational changes in OrgB, InvC and/or the amino terminus of SpaO, which facilitates interaction with the membrane integral components of the T3SS. Alternatively, the SPOA1,2–APAR assembly might function simply by inducing proximity between sorting platform components. Intriguingly, the FliH APAR region has previously been shown to interact with the membrane integral export gate protein FliA¹⁷, suggesting that OrgB/FliH may function as a hub, bridging the ATPase, export gate and SpaO/FliM–FliN. How the sorting platform component OrgA—which lacks a clear flagellar homologue—factors into complex assembly remains to be determined. Similarly, the implications of sorting platform assembly for substrate recruitment, dechaperoning and secretion remain unclear. Our structures will support the precise interrogation of sorting platform interactions in the biomechanics of secretion, and the

necessity of the SPOA1,2–APAR interaction makes it a novel target for the design of anti-virulence compounds.

Methods

Bioinformatics. Sequence alignments were performed using Clustal Omega¹⁸ or M-COFFEE¹⁹. Secondary-structure and disorder predictions were performed using the PSIPRED server²⁰.

Molecular biology. PCR was performed using OneTaq (New England Biolabs), Phusion (New England Biolabs) or PfuTurbo (Agilent) as per manufacturer guidelines with oligonucleotides purchased from Integrated DNA Technologies. All mutations or gene fusions were created by overlap extension PCR. Gene sequences from *S. typhimurium* were PCR amplified from the T3SS-competent strains SB300 (wild type; gift from J. Galán) or SB1741 (3 × FLAG::SpaO, silent SpaO L79_{CTG} to L79_{CTA} variant; gift from J. Galán)⁴. The T4 lysozyme (C54T, C97A) sequence was obtained from Addgene plasmid 18111. An additional mutation (D20N) in T4 lysozyme was made to decrease toxicity in *E. coli*²¹, and the terminal three residues were mutated to alanines to decrease conformational entropy. Standard molecular biology protocols were followed to clone sequences of interest into modified pCDFduet or pETduet vectors for expression in *E. coli* or pBAD for expression in *S. typhimurium*. Restriction enzymes and Quick Ligase (New England Biolabs) were used as per manufacturer specifications. *Salmonella* genomic mutants were produced using homologous recombination from SacB-expressing suicide plasmids²². All SpaO and OrgB mutants were prepared on the SB1741 background. FliM and FliN mutants were prepared on the SB300 background.

Protein expression and purification. Constructs were transformed into BL21(DE3)Gold *E. coli* for heterologous expression and protein expression induced mostly as described²³. Specifically, bacteria were grown to an optical density at 600 nm of 0.5–0.6 at 37 °C in LB medium, the cultures were cooled to 18 °C, induced with 250 μM isopropyl-β-D-thiogalactoside and grown overnight at 18 °C. Selenomethionine (SeMet)-substituted protein was produced in the methionine auxotrophic *E. coli* B834(DE3) grown in methionine-free media supplemented with SeMet²³. Uniformly labelled ¹⁵N/¹³C or ²H/¹⁵N/¹³C protein samples were produced by overexpression in isotopically enriched minimal media. Deuterium oxide, ¹⁵N-ammonium chloride, and ¹³C-glucose were obtained from Cambridge Isotope Labs.

After induction overnight at 18 °C, cells were harvested by centrifugation and resuspended in lysis buffer (200 mM NaCl, 20 mM Tris-Cl pH = 8.0, 5% v/v glycerol, 3 mM imidazole-Cl pH = 8.0, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride and 0.1 mg ml⁻¹ DNaseI). Cells were lysed by 1–2 passes through a mechanical homogenizer (Avestin C5) at 4 °C.

Proteins were purified from *E. coli* cell lysates under native or denaturing conditions (as indicated for each downstream application below) and affinity purified on NiNTA resin (Qiagen). For purification under native conditions, all steps were performed at 4 °C. Lysate was clarified by centrifugation for 30 min at 30,000g and loaded onto NiNTA resin by gravity flow. The column was washed with 5–10 volumes of wash buffer (200 mM NaCl, 20 mM Tris-Cl pH = 8.0, 5% v/v glycerol, 30 mM imidazole-Cl pH = 8.0) and then eluted in elution buffer (200 mM NaCl, 20 mM Tris-Cl pH = 8.0, 5% v/v glycerol, 360 mM imidazole-Cl pH = 8.0). The elution was supplemented with 1 mM EDTA and dialyzed overnight against 200 mM NaCl, 20 mM Tris-Cl pH = 8.0 and 1 mM dithiothreitol. Affinity tags were removed by cleavage with HRV 3C protease.

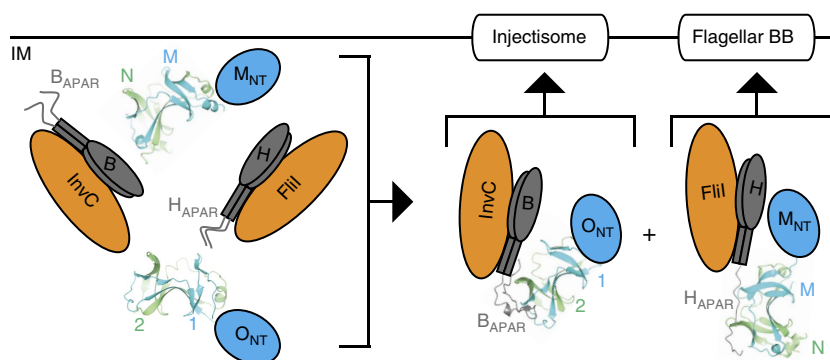


Figure 8 | Subtype-specific assembly of the T3SS sorting platform by SPOA1,2–APAR interactions. Schematic illustration of the proposed role for the SPOA1,2–APAR assembly in organizing and localizing the T3SS sorting platforms in a subtype-specific fashion. IM indicates the inner membrane; O_{NT}, the SpaO amino-terminal domain(s); 1 and 2, SpaO SPOA1 and SPOA2; B, OrgB; H, FliH; M_{NT}, the FliM amino-terminal domains; M and N, the SPOA domains of FliM and FliN; injectisome, the membrane integral components of the pathogenic T3SS; flagellar BB, the flagellar basal body and associated integral membrane components.

For purification under denaturing conditions, guanidinium chloride was added to the lysate to a final concentration of 6 M. The post-extraction lysate was clarified by centrifugation at 30,000g for 15 min at 4 °C and loaded onto NiNTA resin in a batch at 25 °C. Still at 25 °C, the resin was washed with denaturing wash buffer (8 M urea, 500 mM NaCl, 20 mM Tris-Cl pH = 8.0 and 30 mM imidazole-Cl pH = 8.0) and eluted in denaturing elution buffer (8 M urea, 200 mM NaCl, 20 mM Tris-Cl pH = 8.0 and 360 mM imidazole-Cl pH = 8.0). The elution was supplemented with 5 mM EDTA and 5 mM dithiothreitol (DTT), and the protein refolded by dialysis against 200 mM NaCl, 20 mM Tris-Cl pH = 8.0 and 1 mM DTT (3–4 changes, dialysis time of 24 h total, 4 °C). For T4 lysozyme fusions, Hepes-Na pH = 7.0 was substituted for Tris-Cl pH = 8.0. Insoluble material was removed by centrifugation or filtration and affinity tags were removed by cleavage with HRV 3C protease.

Affinity-purified proteins were further purified by ion-exchange chromatography using an AKTA FPLC and the following columns (GE Healthcare): T4 lysozyme fusions were purified by cation exchange on a SourceS column; all other constructs were purified by anion exchange on a SourceQ column. For cation-exchange chromatography, proteins were loaded in a batch in 10 mM Hepes-Na pH = 7.0, 50–100 mM NaCl and eluted by a NaCl gradient (from 0 to 1,000 mM) in the same buffer. For anion exchange, proteins were loaded in a batch in 20 mM Tris-Cl pH = 8.0, 50–100 mM NaCl and eluted by a NaCl gradient (from 0 to 1,000 mM) in the same buffer.

Prior to crystallization, ion-exchange-purified proteins were further purified by gel filtration chromatography on a Superdex 75 column (GE Healthcare) in final buffer (200 mM NaCl, 20 mM Tris-Cl pH = 8.0 and 2 mM DTT) and concentrated using centrifugal concentrators (Amicon). To form the SpaO–OrgB:lysozyme complex for crystallization, cation-exchange-purified OrgB(1–30):T4 lysozyme was mixed with an excess of anion-exchange-purified SpaO(145–213) + SpaO(232–297) and allowed to incubate overnight at 4 °C. The SpaO–OrgB:lysozyme complex was then purified by gel filtration chromatography. To form the FliM:FliN–FliH:lysozyme complex for crystallization, anion-exchange-purified FliM(245–334):FliN(5–137) was mixed with an excess of cation-exchange-purified FliH(1–18):T4 lysozyme and allowed to incubate overnight at 4 °C. The FliM:FliN–FliH:lysozyme complex was then purified by gel filtration chromatography.

Crystallization. All proteins were crystallized by hanging-drop vapour diffusion with 1:1 and 2:1 ratios of protein (in final buffer) to precipitant at 25 °C (except where noted). For crystallization, SpaO(232–297) and FliM(245–334):FliN(5–137) were purified under native conditions; SpaO(145–213) and SpaO(232–297) were purified under denaturing conditions and co-refolded; the T4 lysozyme fusions were purified under denaturing conditions, refolded and mixed with their cognate SPOA1,2 as described above. The protein concentrations, crystallization buffers and cryoprotection conditions for each protein or complex are as follows:

SpaO(232–297) was concentrated to 8 mg ml⁻¹ and crystallized with 35% PEG400, 200 mM calcium acetate and 100 mM sodium acetate pH = 5.0. Crystals were cryoprotected in the mother liquor. Microseeding was performed to enhance crystal uniformity and diffraction. Briefly, crystals to be seeded were harvested in precipitant solution and vortexed in a microfuge tube with a small stir bar for ~60 s. The slurry of microseeds was serially diluted (5–10-fold steps) in precipitant solution and five selected microseed precipitant mixtures were mixed with fresh protein as in a normal hanging-drop experiment.

SpaO(145–213) + SpaO(232–297) was concentrated to 12 mg ml⁻¹ and crystallized with 25% PEG400, 10% isopropanol and 100 mM sodium citrate pH = 5.6 at 4 °C. Microseeding (as above) was performed to enhance crystal uniformity and diffraction. Crystals were cryoprotected in mother liquor with the PEG400 concentration raised to 37.5%.

SpaO(145–213) + SpaO(232–297) + OrgB(1–30):T4 lysozyme was concentrated to 18.5 mg ml⁻¹ and crystallized with 25% PEG3350, 200 mM ammonium formate and 100 mM sodium acetate pH = 5.0. Microseeding (as above) was performed to enhance crystal uniformity and diffraction. Crystals were cryoprotected in 30% PEG3350, 10% glycerol, 200 mM ammonium acetate and 100 mM sodium acetate pH = 5.0.

SpaO(145–213, SeMet) + SpaO(232–297, SeMet) + OrgB(1–30):T4 lysozyme (native) was concentrated to 18 mg ml⁻¹, supplemented with 50 mM maltose and crystallized with 25% PEG3350, 200 mM ammonium formate and 100 mM sodium acetate pH = 5.0. Microseeding (as above) was performed to enhance crystal uniformity and diffraction. Crystals were cryoprotected in 25% PEG3350, 10% ethylene glycol, 200 mM ammonium formate, 100 mM sodium acetate pH = 5.0 and 50 mM maltose.

FliM(245–334):FliN(5–137) was concentrated to 7.5 mg ml⁻¹ and crystallized with 2.2 M NaCl and 100 mM imidazole-Cl pH = 8.0. Crystals were cryoprotected with 2 M NaCl, 100 mM imidazole-Cl pH = 8.0 and 30% glycerol.

FliM(245–334):FliN(5–137) + FliH(1–18):T4 lysozyme was concentrated to 17 mg ml⁻¹ and crystallized with 11% PEG400 and 100 mM sodium potassium phosphate pH = 6.5. Crystals were cryoprotected with 40% PEG400 and 200 mM sodium potassium phosphate pH = 6.5.

Structure determination. Data were collected at the National Synchrotron Light Source (Brookhaven National Laboratory) beamline X29A at a temperature of

– 173 °C using the following X-ray wavelengths: 0.979 Å for SeMet crystals, 1.075 Å for native crystals. Diffraction data sets were indexed and integrated in iMOSFLM²⁴ and scaled and reduced with AIMLESS²⁵. Data sets were truncated at $I/\sigma I > 2.0$, and all sets were determined to have a $CC_{1/2} > 0.7$ in the outermost resolution shell²⁶ (Tables 1 and 2).

The PHENIX program suite²⁷ was used to solve the crystallographic phase problem. SpaO(232–297), SpaO(145–213) + SpaO(232–297) and FliM(245–334):FliN(5–137) were solved by SeMet single-wavelength anomalous diffraction in Autosol. The SPOA1,2–APAR:lysozyme structures were solved by molecular replacement in Phaser-MR using the experimentally phased cognate SPOA1,2 structure and T4 lysozyme (PDB 2LZM). Structures were built in Phenix (Autobuild) with additional manual model building performed in Coot²⁸.

Structures were refined and validated in Phenix (Tables 1 and 2). SpaO(145–213) + SpaO(232–297) + OrgB(1–30):T4 lysozyme crystals exhibited twinning and were refined in Phenix using the twin law 1, –k, h. Ramachandran statistics for all models are as follows: SpaO(232–297, SeMet): 98% favoured, 0% outliers; SpaO(145–213) + SpaO(232–297): 89% favoured, 3% outliers; SpaO(145–213) + SpaO(232–297) + OrgB(1–30):T4 lysozyme: 94% favoured, 0.8% outliers; SpaO(145–213, SeMet) + SpaO(232–297, SeMet) + OrgB(1–30):T4 lysozyme: 89% favoured, 1.8% outliers; FliM(245–334):FliN(5–137, SeMet): 92% favoured, 0.9% outliers; FliM(245–334):FliN(5–137) + FliH(1–18):T4 lysozyme: 94% favoured, 0.9% outliers.

ANODE²⁹ was used to perform *post hoc* analysis of anomalous scatterers in SpaO(145–213, SeMet) + SpaO(232–297, SeMet) + OrgB(1–30):T4 lysozyme crystals, providing additional empirical support for the SpaO–OrgB model coordinates (Supplementary Fig. 6b). Except where indicated, all representations of models and maps for figures were produced in QtMG³⁰.

NMR spectroscopy. The NMR sample of refolded SpaO(140–297) consisted of 0.3 mM U-2H₂¹⁵N/¹³C-labelled protein in 10 mM citrate buffer at pH 5.6 with 90% H₂O/10% D₂O (v/v), 100 mM NaCl and 1 mM dithiothreitol. For comparison of the apo and APAR-bound forms, ¹⁵N/¹³C-labelled SpaO(140–297) was co-refolded with an excess of unlabelled thioredoxin:OrgB(2–30). The thioredoxin solubilization tag was cleaved off by overnight incubation with HRV 3C protease. Protease and affinity tags were removed on NiNTA resin and the SpaO–OrgB complex was separated from the majority of free thioredoxin by Superdex 75 gel filtration chromatography. The final concentration of the protein complex was 0.2 mM in 10 mM citrate buffer at pH 5.6 supplemented with 10% v/v deuterium oxide, 100 mM NaCl and 2 mM dithiothreitol.

The NMR data were collected on Bruker 600, 800 and 900 MHz AVANCE spectrometers equipped with TCI/TXI CryoProbes at 20 °C for the apo-SpaO and 30 °C for the APAR-bound forms. For resonance assignments of apo-SpaO, transverse relaxation-optimized triple-resonance³¹ experiments including trHNCO, trHN(CA)CO, trHNCA, trHN(CO)CA, trHNCACB and trHN(CO)CACB were acquired at 600 and 900 MHz. A three dimensional ¹⁵N-edited nuclear Overhauser enhanced spectroscopy-heteronuclear single quantum coherence spectrum with 100 ms mixing time was also acquired at 900 MHz. To assign APAR-bound SpaO, a suite of conventional backbone experiments³² were acquired at 600 and 800 MHz.

The data were processed in Topspin 2.1 spectra and analysed using the AtoLink module in CARA 1.5 (ref. 33). In both apo-SpaO and its complex with APAR, we were able to successfully assign >95% of the backbone resonances. The heteronuclear chemical shifts were analysed using the TALOS+³⁴ database to predict the secondary structure of the protein. The weighted CSDs were calculated from amide proton (H) and nitrogen chemical shifts (¹⁵N) using the following equation: $CSD = \sqrt{((\Delta\delta H)^2 + ((\Delta\delta^{15}N)/5)^2)}$

Co-affinity purification assay. For co-affinity purification of the SpaO–OrgB–InvC complex (Figs 3 and 5), the proteins indicated were co-expressed and purified under native conditions as described above. For the SPOA–APAR:lysozyme pull-down experiment (Fig. 6), the indicated SPOA-containing proteins were Ni-affinity purified under native conditions, their affinity tags were removed by overnight incubation with HRV protease 3C and they were further purified by anion-exchange chromatography (as above). APAR:lysozyme fusions were separately purified under denaturing conditions and were subjected to cation-exchange chromatography after refolding (as above). One mg of hexahistidine-tagged APAR:lysozyme fusion protein was mixed with 2 mg of the indicated SPOA-containing protein in 0.2 M NaCl and 20 mM Tris-Cl pH = 8.0 (final volume 4 ml) and incubated on ice for 2 h. The mixture was passed twice over 2 ml of NiNTA resin, washed with 8 ml wash buffer (200 mM NaCl, 20 mM Tris-Cl pH = 8.0, 5% v/v glycerol and 30 mM imidazole-Cl pH = 8.0) and then eluted in 3.5 ml elution buffer (200 mM NaCl, 20 mM Tris-Cl pH = 8.0, 5% v/v glycerol and 360 mM imidazole-Cl pH = 8.0).

In vitro secretion assay. *S. typhimurium* of the indicated genotype were grown for 6 h at 37 °C in LB medium with NaCl supplemented to a final concentration of 0.3 M. Cells were pelleted by centrifugation at 3,400g for 0.5–1 h and the supernatants were 0.22-µm filtered. Secreted proteins were precipitated from the filtered supernatants with 15% trichloroacetic acid overnight at 4 °C. The precipitate was

pelleted by centrifugation at 3,400g for 1 h at 4 °C, resuspended in ice-cold acetone and transferred to a microfuge tube. After 0.25 h on ice, the precipitate was harvested by centrifugation at 16,000g for 0.75 h at 4 °C and resuspended in 0.2 M Tris-Cl pH = 8.0 and 0.2 M NaCl to neutralize any residual acid before the addition of SDS-polyacrylamide gel electrophoresis loading buffer. For plasmid complementation analysis, *S. typhimurium* were electroporated with SpaO sequences cloned into the pBAD vector and expression was induced with 0.01% arabinose for the entire duration of the experiment.

Fluorescence microscopy. *S. typhimurium* were grown as for the *in vitro* secretion assay. Cells were harvested by centrifugation, washed three times in PBS and fixed overnight with 4% formaldehyde in PBS at 4 °C. Cells were again washed three times in PBS, counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich) and 10 mM Nile Red (Sigma-Aldrich), and immobilized on poly-L-lysine (Sigma-Aldrich)-coated coverslips. Covers were mounted in Prolong Diamond (Life Technologies) and sealed with nail polish. Slides were imaged on a DeltaVision Image Restoration Microscope with a $\times 100$ objective (Applied Precision). Images were deconvoluted in Softworx (Applied Precision) and processed identically in ImageJ (NIH) and Photoshop (Adobe).

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Acknowledgements

We would like to thank Deena Oren and Alison North (Rockefeller University), and Wuxian Shi (Brookhaven National Laboratory) for technical assistance and discussions. We are grateful to Matthew Lefebvre (Yale University) and F. Nina Papavasiliou (Rockefeller University) for discussions. Jorge Galán (Yale University) graciously donated plasmids and *S. typhimurium* strains. This work was funded in part by NIH grants to C.E.S. (AI52182 and AI093704) and research funds from the Rockefeller University. R.Q.N. was supported by the Hearst Foundation and by a Medical Scientist Training Program grant from the National Institute of General Medical Sciences of the National Institutes of Health under award number T32GM07739 to the Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program. X-ray diffraction data for this study were measured at beamline X29A of the National Synchrotron Light Source. Financial support comes principally from the Offices of Biological and Environmental Research and of Basic Energy Sciences of the US Department of Energy, and from the National Institute of General Medical Sciences (P41GM103473) and the National Institute of Biomedical Imaging (P30-EB-00998). Preliminary X-ray diffraction experiments performed on the Rigaku/MSO microMax 007HF in the Rockefeller University Structural Biology Resource Center were made possible by Grant Number 1S10RR022321-01 from the National Center for Research Resources of the NIH. NMR data were collected at the New York Structural Biology Center, made possible by a grant from NYSTAR and ORIP/NIH facility improvement grant CO6RR015495. The 900-MHz NMR spectrometers were purchased with funds from NIH grant P41GM066354, the Keck Foundation, New York State Assembly and the US Department of Defense. The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions

R.Q.N. conceived of the project, designed and performed experiments, analysed data and wrote and revised the paper. S.B. designed and performed experiments, analysed data and revised the paper. M.L. performed experiments and revised the paper. C.E.S. conceived the project, analysed data and wrote and revised the paper.

Additional information

Accession codes: Atomic coordinates have been deposited in the Protein Data Bank under the following deposition codes: 4YX1, SpaO(232–297, SeMet); 4YX5, SpaO(145–213) + SpaO(232–297); 4YX7, SpaO(145–213) + SpaO(232–297) + OrgB(1–30)::T4 lysozyme; 4YXA, SpaO(145–213, SeMet) + SpaO (232–297, SeMet) + OrgB(1–30)::T4 lysozyme (native); 4YXB, FliM(245–334)::FliN(5–137)

SeMet; 4YXC, FliM(245–334)::FliN(5–137) + FliH(1–18)::T4 lysozyme. NMR chemical shifts are deposited in the BMRB under ID 26543 (apo-SpaO) and 26546 (APAR-bound SpaO).

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Notti, R.Q. *et al.* A common assembly module in injectisome and flagellar type III secretion sorting platforms. *Nat. Commun.* 6:7125 doi: 10.1038/ncomms8125 (2015).



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