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Structural control for the coordinated assembly into

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functional pathogenic type-3 secretion systems

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39 Abstract

40 Functional injectisomes of the type-3 secretion system assemble into highly defined and
 41 stoichiometric bacterial molecular machines essential for infecting human and other
 42 eukaryotic cells. However, the mechanism that governs the regulated step-wise assembly
 43 process from the nucleation-phase, to ring-assembly, and the filamentous phase into a
 44 membrane embedded needle complex is unclear. We here report that the formation of a
 45 megadalton-sized needle complexes from *Salmonella enterica* serovar Typhimurium
 46 (SPI-1, *Salmonella* pathogenicity island-1) with proper stoichiometries is highly
 47 structurally controlled competing against the self-assembly propensity of injectisome
 48 components, leading to a highly unusual structurally-pleiotropic phenotype. The structure
 49 of the entire needle complex from pathogenic injectisomes was solved by cryo electron
 50 microscopy, focused refinements (2.5-4 Å) and co-variation analysis revealing an overall
 51 asymmetric arrangement containing cyclic, helical, and asymmetric sub-structures. The
 52 centrally located export apparatus assembles into a conical, pseudo-helical structure and
 53 provides a structural template that guides the formation of a 24-mer cyclic, surrounding
 54 ring, which then serves as a docking interface comprising three different conformations
 55 for sixteen N-terminal InvG subunits of the outer secretin ring. Unexpectedly, the secretin
 56 ring excludes the 16th protein chain at the C-terminal outer ring, resulting in a pleiotropic
 57 16/15-mer ring and consequently to an overall 24:16/15 basal body structure. Finally, we
 58 report how the transition from the pseudo-helical export apparatus into the helical
 59 filament is structurally resolved to generate the protein secretion channel, which provides
 60 the structural basis to restrict access of unfolded effector substrates. These results

highlight the diverse molecular signatures required for a highly coordinated assembly process and provide the molecular basis for understanding triggering and transport of unfolded proteins through injectisomes.

Keywords

Type-3 secretion system, nanomachine, assembly, cryo electron microscopy, infection

Introduction

Molecular machines ^{1,2} can only perform their function in the cell if they assemble properly ³. Hence, mis-assembly into non-functional or defective systems is energetically costly and could even result in unwanted side-effects to the cell. As a consequence, assembly processes of complex molecular machines composed of one or many copies of numerous components are tightly regulated including various control elements at the transcriptional, translational, post-translational or structural level. These elaborate controls ensure a spatiotemporal confined and defined order of assembly steps into functional complexes, such as the ribosome or the spliceosome, executed in a crowded cellular environment ^{4,5}.

Type-3 secretion systems (T3SSs) assemble at bacterial membranes into syringe-like nano-machines that are critical for bacterial infection by many human pathogens including *Salmonella*, *Shigella*, *Yersinia* or *EPEC* (enteropathogenic *E. coli*). They actively deliver toxic bacterial effector proteins into their respective host cells ⁶. As a consequence, T3SS-mediated infection can be prevented by interfering with the

82 functionality of the T3SS ⁷. The core of the T3SS (SPI-1, *Salmonella* pathogenicity
83 island-1) is the needle complex (NC) ⁸, a membrane-embedded structure that mediates
84 contact between bacterial and eukaryotic cells and contains a shielded secretion path for a
85 safe delivery of unfolded effector proteins ^{9,10}. The needle complex is intricately
86 assembled from multiple proteins (Supplementary Table 7) in a series of stacking
87 membrane rings (PrgH, PrgK, InvG) to form a ‘basal body’ from which a helical needle
88 filament (PrgI) extends into the environment ^{11,12}. The basal body encompasses the
89 centrally located export apparatus (SpaP, SpaQ, SpaR, SpaS, InvA), which defines the
90 entry of the secretion path into the secretion channel ¹⁰ and continues into the filament by
91 attaching it with the inner rod protein PrgJ. All these proteins are thus essential for T3SS
92 function ^{7,13}. Recent structural investigation of the evolutionary related export apparatus
93 from the flagellar system revealed a pseudo-helical arrangement of a ternary sub-complex
94 ^{14,15} (FliP, FliQ, FliR (flagellar system)/SpaP, SpaQ, SpaR (injectisome), Supplementary
95 Table 7). The FliPQR complex has been recombinantly produced in the absence of the
96 flagellar context and the unusual packing of the individual protein chains, which all have
97 been bioinformatically predicted as integral membrane proteins, together with the
98 previous observation that the PQR-subcomplex precedes basal body/needle complex
99 assembly (**‘initiation/nucleation phase’**) into functional pathogenic T3SSs (Figure 1a) ¹³
100 raises the question, as to how this is mechanistically controlled. Notably, ring formation
101 of the inner (IR; PrgH, PrgK) and outer rings (OR; InvG) and their interaction into
102 stacked rings (**‘ring-phase’**) to generate non-functional basal-body-like complexes can
103 also occur in the absence of export apparatus proteins, suggesting that this processes is

self-sufficient¹³. Furthermore, the observation that the oligomericity of rings in the flagellar system can vary¹⁶, similar to earlier reports of different ring-diameters in pathogenic T3SSs¹⁷, together with increasingly high-resolution structural investigations of a defined oligomericity of the OR (15-mer) and the IR (24-mer)^{11,12,18}, challenges how the OR and IR specifically interact and whether and how oligomericity is controlled. Ultimately, an answer to this conundrum would clarify whether a structurally singular or diverse group of physiologically relevant T3SSs exists.

Assembly of needle complexes is finalized by the growth and attachment of the filament to the export apparatus (**‘filamentous phase’**, Figure 1a). This phase is already dependent on the secretion activity of the T3SS, which transports protomers of PrgJ and PrgI into the chamber of the basal body, where they assemble into a filamentous structure that connects to the export apparatus within and extends as a PrgI-only helical filament projecting away from the basal body^{6,17,19}. Recent work has identified PrgJ residues that are in the vicinity of the export apparatus and a single helical turn of PrgJ has been suggested to serve as an adaptor to the filament²⁰. Nevertheless, it remains largely unknown, how monomers of PrgJ and PrgI are packaged into a quaternary structure and attached to the export apparatus, which is critical to form the majority of the protein secretion channel within the basal body.

To dissect the coordinated process of the directionality of the assembly phases into needle complexes of pathogenic T3SSs and to understand the molecular signatures promoting high-order assembly and stability, we investigated the cryo electron microscopy structures of the entire wild-type (WT) and export-apparatus (EA) deletion mutant

complexes from *Salmonella enterica* serovar Typhimurium to 2.5-4 Å resolution and performed extensive mutational and functional analyses. We show that (1) oligomericity of the rings is controlled by the nucleating SpaPQR complex of the export apparatus, (2) an unusual structural pleiotropy is crucial for inter- and intra-ring stability, and (3) the synthesis of the intra-basal secretion channel is the consequence of a remarkable packing of the asymmetric export apparatus to the symmetrically helical filament. The fact that without a coordinated assembly line, highly structured basal body-like complexes can be assembled, but with a diverging quaternary architecture leading to non-functionality, emphasizes the necessity and importance of flawless assembly processes for molecular machines in general.

Methods

All relevant materials and methods are described in the supplementary documents (genetic manipulations, secretion assays, biochemical purifications, co-variation analyses, electron microscopy and analyses, sample vitrification and cryo electron microscopy, single particle and helical reconstructions, model building, model refinement and model validation, visualization, deposition).

146 **Results**

147 **The structure of the entire needle complex**

148 We first purified WT needle complexes¹⁰ from *Salmonella*, and vitrified the sample on
 149 holey carbon grids containing a layer of either continuous carbon or a freshly made
 150 graphene oxide as a sample support (Supplementary Table 3). This procedure allowed us
 151 to image particles at a wider angular distribution (Supplementary Figure 5) during cryo
 152 electron microscopy. We subsequently determined the structure of the entire needle
 153 complex by single particle analysis. Due to the size and the flexibility of the needle
 154 complex¹¹ we applied focused refinements to various parts of the complex and resolved
 155 individual sub-structures to resolutions ranging from 2.6 to 4 Å (Figure 1b). While
 156 overall the needle complex is an asymmetric molecule, individual parts of the complex
 157 display non-symmetric (C1), cyclic (C8, C15, C18) and helical symmetries.

158

159 **The export apparatus proteins dictate inner ring symmetry**

160 Both, the IR (PrgH/PrgK) and the OR (InvG) are highly oligomeric rings that are able to
 161 self-assemble into a basal body even in the absence of the export apparatus¹³. Because of
 162 the tight and highly stable arrangement of InvG into a 60-strand beta-barrel and its ability
 163 to independently form the outer ring in the absence of IR components¹⁸, we
 164 hypothesized, whether the symmetry and the assembly of the larger IR with its 24-fold
 165 oligomeric state is governed by the InvG OR. We thus analyzed structures of purified and
 166 vitrified complexes from an InvG knock-out¹³ and compared it to the wild type (WT)

basal body complex. We found that the IR dimension from the InvG knockout is indistinguishable from the WT, suggesting that the 24-fold symmetry as clearly observed in WT from end-on views is maintained (Figure 1c). Thus, the 24-fold IR-ring formation is independent from the presence of the outer membrane protein InvG.

To determine if the centrally located export apparatus (EA) of the needle complex controls IR symmetry, we next visualized complexes isolated from an EA-knockout strain (Δ EA: Δ *spaPQRS*, Δ *invA*). Remarkably, we found that the IR in these complexes is smaller in diameter and contains only 23 subunits, as observed from end-on-views (Figure 1c). To further identify, which components of the export apparatus are responsible for this atypical structural phenotype, we analyzed previously purified and vitrified structures from individual SpaPQR, InvA knock-out strains²¹. We found that structures obtained from an Δ *invA* strain are WT-like, whereas structures recovered from Δ *spaP*, Δ *spaQ*, or Δ *spaR* strains are indistinguishable from the complete EA knock-out (Supplementary Table 1). Interestingly, the complexes obtained from the Δ *spaS* strain could be sub-classified in two phenotypes that either are WT-like (75%) or EA-knock-out-like (25%) (Figure 1d, Supplementary Figure 1, Supplementary Table 1). Together with earlier findings that PQR forms an initial complex during the assembly¹³, our observations lead us to propose a model that the PQR sub-complex generates a structural template for the formation of precisely 24-subunits of PrgH and PrgK, respectively, for the complete IR assembly.

The structure of the export apparatus provides a structural template for needle complex assembly into functional injectisomes

The structure of the EA bound in the center of the needle complex reveals a conical shape with a pseudo-helical arrangements of individual protein chains, displaying a P:Q:R = 5:4:1 stoichiometry (Figure 2, Supplementary Figure 8, Supplementary Table 5). The sub-structure is structurally similar to reconstituted samples of the flagellar export apparatus¹⁵. The export apparatus proteins SpaP, SpaQ, SpaR, and SpaS have been bioinformatically predicted as membrane proteins containing alpha-helical hydrophobic stretches suggesting a canonical lateral organization in a lipid bilayer. However the spiral arrangement of 4 consecutive SpaQs at the bottom of the EA sub-structure generates a cork-screw-like hydrophobic belt (Figure 2c) consistent with the idea that SpaQ is in part lifted out of the membrane during the assembly¹⁵. A single chain of SpaR intersects the assembly of 5 successive SpaPs (Figure 2b), which conclude the upper part of the EA with a hydrophilic, acidic belt, indicating that these proteins do not behave as classical integral membrane proteins. The separation of the EA into two surrounding belts, different in their surface properties, demarcates its position relative to the surrounding PrgK ring (Figure 2c, d). At the corresponding position of the PrgK ring are two complementary enclosures established by a lower and conserved, largely hydrophobic (aspartate-92 to proline-98, Figure 2d,e (arrow)) and a higher hydrophilic loop (glycine-137 to proline-142) that project towards the EA. Starting from the bottom tip of the PQR conus, the PQR complex is slightly located off center relative to the projected center determined by the PrgK ring. From there, it spirals upwards and establishes hydrophobic

211 contacts primarily to the lower PrgK-loop (Figure 2d, lower, Supplementary Figure 2).
 212 Subsequent interactions to PrgK are determined by the curvature of the PQR-complex
 213 and are captured largely within the first half-circle of PrgK, explaining how the PQR
 214 complex provides a structural template for ring assembly. The interaction to PrgK is
 215 further stabilized by contacts of SpaP, which constitutes the upper part of the EA.
 216 Interestingly, the fourth component, SpaS, is not visible in the reconstructions suggesting
 217 that it has been largely stripped off during the purification. This is in agreement with
 218 earlier biochemical data ¹³. Nevertheless, because SpaS only fractionally influences the
 219 efficiency of proper ring assembly, we considered that SpaS is localized at the PQR
 220 complex in the vicinity of the PrgK ring position. We thus performed a co-variation
 221 analysis, showing that indeed the strongest signals are obtained between SpaS-SpaQ and
 222 SpaS-SpaR (Supplementary Table 2). We used this information together with a template
 223 structure from a flagellar EA complex (unpublished data, S. Lea) to model SpaS onto the
 224 PQR complex (Figure 2f), demonstrating that the majority of SpaS is positioned along a
 225 conserved patch of the hydrophobic belt (Figure 2g) and surrounds the tip of the
 226 hydrophilic PQR conus. However, two SpaS loops reach into the conserved hydrophobic
 227 PrgK enclosure, thereby completing a circular packing of PQRS in the center of the PrgK
 228 ring (Figure 2d).
 229 Taken together, the structure of the EA explains how the PQR/S-sub-structure can serve
 230 as a structural template for inner ring formation into physiologically relevant 24-mer
 231 complexes. This is also consistent with earlier biochemical observations that the export
 232 apparatus, in particular PQR, followed by PQRS forms a sub-structure that initiates

assembly into full WT basal body¹³. Our structure of the EA within fully assembled injectisomes also clarifies that the majority of PQR (and S) monomers are embedded outside a membrane bilayer and thus represent a class of molecules that either can be membrane proteins during assembly or soluble proteins within the complex.

Active injectisomes are asymmetric and structurally pleiotropic

Entire needle complexes are structurally complex due to their size, their stoichiometry and copy-number of polypeptide chains, their various symmetries (cyclic, helical) and asymmetry (pseudohelical conus) in sub-parts of the injectisome. As a consequence, many interactions at and between various parts are necessary to establish sufficient stability but also conformational flexibility, essential for assembly¹⁷. To address the relevant factors for proper needle complex assembly, we first analyzed the interaction of the larger inner (PrgH/K) and smaller outer membrane ring (InvG) by focused reconstructions at the interface of the IR and OR (Figure 3, Supplementary Figures 9-10). We found that 16 out of 24 PrgH monomers tightly associate with their ~30 amino acid C-terminal tails to 16 subunits of the N-terminal InvG-ring (domains N0-N1). They form 8x2 beta-sheets (conformation ‘B’ and ‘C’ in PrgH) that intercalate between 16 beta-stranded InvG domains and thus establish together with InvG a large circular and flat beta-stranded ring facing the 24-mer PrgH/K ring (Figure 3a). Intercalation and formation of the C-terminal beta-sheet is essential to confer stability between the rings (Figure 3b) and explains previous functional data of truncated PrgH^{22,23}. Furthermore, the remaining 8 subunits of PrgH also form a short two-stranded beta-sheet (conformation ‘A’), which

255 binds to the exterior part of InvG as well as to a neighboring PrgH and presumably
256 stabilizes the overall arrangement.

257 The 16-mer structure of the secretin InvG is in sharp contrast to previous high resolution
258 structures that reported 15 subunits for this domain ¹². Interestingly, only the upper, C-
259 terminal domain of InvG maintains a 15-mer ring, as shown in our 2.6 Å resolution
260 structure (Supplementary Figure 11, Table 4) and similar to earlier reports ¹⁸. This raises
261 the question as to how the individual InvG monomers that start with their N-terminal
262 domain linked to the larger inner ring span the periplasm and extend into a the 15-mer
263 upper OM ring. We thus performed focused reconstructions in C1 of the full OR (Figure
264 3c). At first, we found that the upper and lower domains of the OR are slightly tilted and
265 that the continuous density bridging the constricted ‘neck-region’ (labelled in Figure 2a)
266 is largely present only on one side of the neck. Notably, we found that indeed, the entire
267 OR assembles into a lower 16-mer and an upper 15-mer ring, suggesting that the upper
268 part of the 16th subunit is left out during the 15-mer ring assembly. Moreover, we found
269 that the 16th InvG monomer is cleaved in isolated needle complexes, as demonstrated by
270 the detection of a faster migrating band in Western blots using an antibody raised
271 exclusively against the lower InvG-ring domains (N0-N1, Figure 3d). To test, whether the
272 lower OR, represented by the N0-N1 domains of InvG, remains stable as a ring in the
273 absence of the larger IR, as an indicator for stacking of preformed rings²⁴, we established
274 a mild disassembly protocol that selectively removes the IR components. Surprisingly,
275 we found by cryo electron microscopy that the lower OR (N0-N1) becomes largely
276 unstructured and the individual domains very flexible, whereas the upper secretin ring

maintains as a stable ring (Figure 3e). Similar observations have been reported upon isolation of only secretin-rings¹⁸. This demonstrates that the so-called ‘ring-forming’ domains N0-N1¹² in the lower part of InvG are in fact unable to independently form stable rings and require the presence of the larger 24-mer inner membrane ring for (16-mer) ring formation. Consequently, we speculated, whether the stability of the entire secretin ring is largely mediated by the overall structural arrangement of the upper InvG ring. While the majority of the individual monomers are arranged side-by-side, generating a beta barrel with 60 strands tilted by +33°, a long C-terminal stretch (amino acids: V519-G557) interacts as an extended conformation at approximately -40° with two neighboring InvG monomers in the opposite direction (n-1, n-2) of the tilted strands (Figure 3f). To test, whether the C-terminal end of InvG indeed provides stability for upper ring formation, we generated C-terminal truncation mutants, probed for functionality and analyzed complex formation (Figure 3g). We found that truncations of only six amino acids already impacted, and ten amino acids that eliminate only a fraction of the very last alpha helix of InvG, completely abrogated needle complex formation and function (Figure 3g, Supplementary Figures 3 and 6).

Taken together, the InvG ring in functional needle complexes is structurally pleiotropic, displaying different stoichiometries in the upper and lower OR. The assembly of the secretin into functional needle complexes requires 16 monomers at the basal side to interact with 8x2 intercalating PrgH C-terminal domains (CTD) at the inner membrane. It is furthermore stabilized by binding of 8 additional ‘exterior’ PrgH-CTD’s, and 15 InvG protomers to form a stable ring at the apical side that connects to the outer membrane.

299

300 **The atomic structure of a dead-end T3SS**

301 The fact that complexes obtained from EA knock-outs exclusively adopt a 23-mer
 302 arrangement in the larger IR (Figure 1c) from otherwise native PrgH/PrgK proteins raises
 303 the question, as to how basal-body formation is structurally controlled. In order to gain
 304 detailed structural insight into the mechanism by which assembly into aberrant
 305 injectisomes diverges from assembly into functional systems, we determined the atomic
 306 structure of the export apparatus knock-out T3SS (Figure 4). We thus applied a similar
 307 purification strategy and observed by negative-stain electron microscopy that the entire
 308 Δ EA needle complex but also complexes isolated from strains of individual knock-outs
 309 for SpaP, SpaQ, and SpaR are less stable (Supplementary Figure 7). Very often, the larger
 310 inner and smaller outer membrane rings were displaced from the complexes.
 311 Interestingly, this behavior was largely absent upon vitrification, indicating that negative
 312 staining induces sufficient shear forces that induce structural deviations in isolated knock-
 313 out but not WT complexes. We solved the cryo electron microscopy structure from the
 314 Δ EA basal-body complex to 2.5-3.4 Å (C23; C1) resolution (Table 4). Overall, the PrgH/
 315 K ring adopts a 23-mer structure with a similar intertwined arrangement of non-tilted
 316 PrgH and 24° tilted PrgK (Supplementary Figure 4a) that stabilizes the large IR.
 317 Comparison of the interacting surfaces of neighboring (PrgH,PrgK):(PrgH,PrgK)
 318 protomers revealed a larger area in the Δ EA 23-mer than in WT (23-mer: 4526.5 Å²; 24-
 319 mer (WT): 4435.5 Å²).
 320 Overall, the 23-mer ring has a smaller diameter (inner diameter 67Å) than in the WT

complex (inner diameter 73Å), with insufficient space for the EA located at the equivalent position and height relative to the PrgK rings (Figure 4, Supplementary Figure 4b). This together with the previous packing of EA in WT complexes, establishes that the EA plays an essential role during assembly by generating a structural template that mechanistically counteracts the self-assembly propensity into rings and thus ensures formation of 24-mer rings.

To investigate the structural consequence of ring stacking to a 23-mer IR in respect to the interaction with the OM ring, we determined the upper and lower InvG ring from the Δ EA complex by focused reconstructions to a resolution of 2.7 and 2.8 Å, respectively. To our surprise, we found only a 15/15-mer arrangement of the secretin (Supplementary Figures 17 and 18). This is in sharp contrast to the 16/15-mer arrangement in WT complexes (Figure 3c). This observation is also consistent with the lack of detection of a faster migrating InvG band in Western blots (Figure 3d), suggesting that overall, the secretin protein InvG is able to adopt different configurations (15/15 and 16/15) dependent on the oligomericity of the larger inner ring. In pursuit of understanding the architecture of how a 23-mer ring interacts with a 15-mer to still being able to form mutant basal bodies, we solved the structure without symmetry enforcement (C1) (Figure 4a, Supplementary Figure 16). Contrary to WT complexes that show a highly organized and repeated pattern at the IR/OR interface (8x conformation of ‘A’, ‘B’, ‘C’, respectively, see Figure 4b), the interface of the Δ EA needle complex is irregular and asymmetric throughout the interaction circle (conformation ‘A’ (7x), ‘B’ (7x), ‘C’ (9x), Figure 4b). Despite the suboptimal structural arrangement of the interaction surface, it is

still surprising that basal body-like complexes can be formed suggesting that avidity also plays a role between ring-stabilization.

Structure of the inner rod and internal filament

The final step for needle complex assembly requires active transport of the inner rod protein PrgJ and the needle filament PrgI, which generate a filamentous sub-structure for the secretion of later substrates by connecting the pseudo-helical packing of the conical EA to the exterior helical needle filament structure (Figure 5a). Both proteins are small (101 residues in PrgJ, 80 residues in PrgI) and predominantly alpha helical in nature. To clarify, how PrgJ and PrgI connect and are arranged within needle complexes, we solved the cryo electron microscopy structure by focused refinements around the central part of the needle complex (Figure 3b, Table 4). We found that PrgJ adopts a two-legged alpha-helical structure with similar lengths (Figure 5b). Six PrgJ subunits are circularly, yet asymmetrically organized (for a single PrgJ only one alpha helix (*) is visible; blue ribbon) and interact with the export apparatus protein SpaP. The arrangement establishes a space that is subsequently occupied by PrgI (yellow), allowing individual monomers to enter between and grow upwards as a helix. The helical parameters of the internal part of PrgI are similar to the reconstructions obtained from the external filament (twist/rise 63.3°/4.4 Å in outer filament, 63.4°/4.3 Å in inner filament, Supplementary Figures 14 and 15). PrgI also establishes contacts to the surrounding InvG secretin ring (Figure 5b), suggesting a firm stabilization of the filament within the basal body. Notably, the first five PrgI monomers (yellow) that are localized between the PrgJ monomers are partly

unstructured at amino acids 1-20 in contrast to later PrgI subunits throughout the helical assembly, suggesting that this is a crucial structure that allows the transition from a non-helical to a helical arrangement to occur.

Secretion of unfolded substrates occurs through the export apparatus and continues into the filamentous sub-structure. We thus analyzed the entire channel and found that it starts with a hydrophilic ring at its conical entry (Figure 5c, arrow). Shortly after, the channel snakes through a highly confined area, to then reach a space of approximately 12 Å in diameter, defined by the upper part of the export apparatus. Then, the channel continues through the filament with a slightly larger diameter (14-16 Å), similar to the dimensions observed in the external helical filament (16 Å). The highly confined areas are consistent with a mechanism that restricts passage of only completely unfolded proteins shortly after entry into the export apparatus¹⁰.

Discussion

Correct and spatio-temporal assembly of molecular machines is a prerequisite for their functioning within the cell. Here we have analyzed the molecular signatures required for the directional assembly of the megadalton-sized needle complex of pathogenic T3SSs and have elucidated requirements to establish stability among the proteins involved. We solved the structure of the prominent needle complex of the T3SS from *Salmonella enterica* serovar Typhimurium composed of > 200 polypeptides by cryoEM including co-variational data.

Our work provides the molecular basis of the requirement to form a functional complex and establishes a framework for a detailed analysis of the highly coordinated and structurally controlled assembly path. Comparison with dead-end complexes including mutational and functional data allows us to propose a model for the three consecutive assembly steps, starting from an initiating nucleation phase, progression through the ring-phase, to finalize the process in the filamentous phase.

We found that a tripartite PQR complex of the export apparatus located centrally within injectisomes generates a structure very similar to isolated and recombinantly produced flagellar export complexes¹⁴. This suggests that the PQR complex can form independently during the initiation/nucleation phase prior to ring-formation (Figure 6), which is also consistent with earlier biochemical findings¹³. A defined stoichiometry of PQR (5:4:1) is essential, as the resulting pseudo-helical, conical structure resembles a template for the lateral packing of exactly 24 monomers of PrgH and PrgK, the components of the larger inner ring. The export apparatus protein InvA is not involved in correct 24-mer ring formation, consistent with its physical location away from the core of the PQR complex revealed by cryo electron tomography¹². However, SpaS increases the efficiency of precise assembly likely through its direct interaction at PQR and its immediate vicinity to PrgK. Indeed, the observation that in the absence of the PQR complex, the IR self-assembles to a 23-mer ring only and subsequently culminates in non-functional complexes, highlights the importance for a precise, structure-based control mechanism.

Similarly, the high propensity for self-assembly of InvG protomers into the upper part of

the OR, mediated by a highly intertwined arrangement of individual InvG monomers, and the inability of the N0-N1 InvG domain to maintain a stable ring structure for subsequent stacking to the larger IR to establish the basal body^{24,25}, cannot be reconciled with the here found and unusual pleiotropic structure of the OR (16/15-mer) in isolated injectisomes. Contrary to a stacking model of pre-formed rings, our data convey that 16 individual InvG protomers first connect with their N-terminal domains to the IR, allowing the distant C-terminal domain of InvG to stabilize into a 15-mer with a tightly interwoven OR 60-stranded beta-barrel. It remains unclear, why the 16th InvG of the OR is excluded from the upper part of the ring, resulting in a 16/15-mer configuration within functional injectisomes. It could, however, be explained by an evolutionary re-adaptation of existing secretin folds for stable ring formation employed in other systems, such as various 15/15-mer type-2 secretion systems²⁶⁻²⁸, yet constrained by the presence of a 24-mer IR. This hypothesis is also in agreement with the fact that InvG is able to adapt a 15/15-mer configuration in isolated Δ EA complexes. Taken together, this corroborates the idea that a coordinated, structurally constrained assembly process contains the self-assembly propensity of the outer ring component.

Finally, the growth of the filamentous sub-structure requires to connect to the export apparatus and to transition from a pseudohelical export apparatus rim to the actual helical needle filament. This transition is accomplished by a highly conserved, elegant packing of the inner rod protein PrgJ to SpaP on one side and to the needle filament proteins, which are packaged in between individual PrgJ protomers on the other side. Polymerization of PrgI protomers leads to elongation of the needle filament that pushes

towards the septum of the OR. Subsequently, a conformational change is required to induce the opening of the septum and to allow the continuation of the needle filament growth into the extracellular space¹². There, it serves as the connecting tube that allows the safe translocation of bacterial effector proteins into host cells. The observation that the PrgI filament structure within and outside of the basal body slightly differs, supports the idea of signal transduction mediated by a conformational change through the filament upon host cell contact²⁹. Therefore, the structure of the entire needle complex presented in this study provides the basis for analyzing the mechanism of activation for secretion of toxic bacterial effectors in the future.

Acknowledgments

This project was supported by funds available to TCM at the Institute of Structural and Systems Biology at the University Medical Center Hamburg-Eppendorf, the Institute of Molecular Biotechnology (IMBA) of the Austrian Academy of Sciences, and the Research Institute of Molecular Pathology (IMP). TCM (and SM) received funding through grant I 2408-B22 furnished by the Austrian Science Fund (FWF). VK was supported by Boehringer Ingelheim Fonds PhD fellowship. High-performance computing was possible through access to the HPC at DESY/Hamburg (Germany). and the Vienna Scientific Cluster (Austria). Electron microscopy was preformed at the CSSB Cryo-EM Multiuser facility (Krios, L120C) and the Vienna Biocenter Core Facilities (Polaris, Morgagni).

451 **Author contributions**

- 452 NGM, VK, MJB, JM, JW, SM, OV, WL, SW, FD, TCM - designed experiments
- 453 NGM, JM, SW - prepared knockouts and mutants
- 454 NGM, VK, MJB, OV, SM, JM - purified injectisomes
- 455 NGM, JM, OV, SM, VK - biochemical tests
- 456 MJB, JW, WL - cryoEM data collection and image data management
- 457 MJB, VK, TCM - single particle and helical image data processing
- 458 VK - Co-variation analysis
- 459 NGM - de-novo building and validation
- 460 MJB, NGM, VK, LK, WL, FD, TCM - model building, refinements and interpretation
- 461 VK - prepared figures
- 462 SL, TCM - supervised model refinements
- 463 TCM – original draft
- 464 all authors – draft review and editing
- 465 TCM - supervised project
- 466

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543 **Figure legends**

544

545 **Figure 1: The export apparatus (EA) is essential for assembly into active complexes**

546 a) Schematic of the overall organization and assembly phases of the T3SS needle

547 complex.

548 b) Overview of the needle complex architecture and identified local symmetries.

c) Absence of the export apparatus components (SpaPQRS, InvA), but not OR components (InvG), results in a 23-fold symmetry of IR. Left column: 2D averages of single particle cryoEM side views of isolated WT, $\Delta invG$ and ΔEA complexes. Central column: Averaged density profile at the IR (dashed lines indicate standard deviation of averaged pixel values). Right column: 2D averages of single particle cryoEM top views of isolated WT and ΔEA needle complexes. The number of IR protomers is indicated.

d) Upper: Averaged density profile from individual knock-out needle complexes. IR diameters are measured as the distance of the minima in the density profile. Graphs of complexes showing a WT-like diameter are colored in grey. Graphs of complexes with a significantly smaller diameter are colored in orange. A reduction of normalized pixel intensity in the central area of the IR plane is visible for the same knock-outs, indicating the absence of the EA.

Lower: Diameters of IR and statistical significance (*). WT-like diameters colored in grey, ΔEA -like diameters colored in orange. Complexes from a $\Delta spaS$ mutant could be classified into, WT-like (type1) and ΔEA -like phenotypes (type2). See also Supplementary Table 1.

Figure 2: The structure of export apparatus within the needle complex reveals a structural template for ring formation of active complexes

a) Central cross-section of asymmetric (C1) single-particle reconstruction data of WT

571 needle complexes. OR: outer ring, IR: inner ring. Export apparatus components are
572 colored in dark grey (SpaQ), yellow (SpaR) and dark cyan (SpaP), respectively. Densities
573 for PrgK are colored in orange.

574 b) Structure of the PQR complex present in needle complexes shown as a ribbon diagram
575 from different views (side, top, bottom). The PQR complex adopts a pseudo-helical
576 arrangement with the sequential order of 4x SpaQ (dark grey), SpaR (yellow), and 5x
577 SpaP. (Top right) Individual protein chains for SpaQ and SpaP, respectively, are
578 structurally similar to each other (top-right corner). RMSD range for SpaQ: 1.3-2.4 Å,
579 RMSD range for SpaP: 1.5-3.8 Å (see Supplementary Table 5).

580 c) The surface of the PQR complex contains a charged (*) and a hydrophobic (**) belt.
581 The electrostatic potential was calculated according Coulomb's law (dielectric constant
582 $\epsilon=4$). The hydrophobicity is colored according to the scale of Kyte and Doolittle³⁰. Side
583 views on the left are rotated around the x-axis to provide bottom-views of the EA on the
584 right.

585 d) Upper: half-cut side view of the PrgK ring shows a hydrophobic and conserved loop
586 facing the interior of IR (arrow). Lower: The hydrophobic belt of the PQR complex binds
587 tightly to the surrounding hydrophobic PrgK ring.

588 e) The loop (aspartate-92 to proline-98) of PrgK is largely hydrophobic and conserved.

589 f) Homology model of SpaS (ribbon) bound to the PQR complex (surface). Evolutionary
590 couplings between SpaS/SpaR and SpaS/SpaQ are shown in cyan and orange,
591 respectively. Notably, SpaS twists in a circle around the conical PQR entry.

592 g) Surface representation of the PQR(S) complex colored by conservation as determined
593 by ConSurf³¹.

594

595

Figure 3: WT needle complexes are asymmetric and structurally pleiotropic

a) Cryo electron microscopy structure of the IR and OR of WT needle complexes.

Twenty four protomers of PrgH and PrgK, respectively, interact with sixteen InvG

600 protomers. The interaction is established by three repeating conformations at the C-
 601 terminal tail (amino acids 361-392, conformation ‘A’, ‘B’, ‘C’) of PrgH resulting in an
 602 extended circular beta-sheet composed of PrgH and InvG. The overall structure displays a
 603 C8 symmetry with 3 PrgH (H1, H2, H3, blue), 3 PrgK (K1, K2, K3, grey), and 2 InvG
 604 (G1, G2, yellow) in the asymmetric unit.

605 b) Successive truncations of amino acids at the PrgH C-terminus impair complex
 606 formation. Deletions of ten amino acids do not yield stable needle complexes as analyzed
 607 by electron microscopy. Scale bar 50 nm. See also Supplementary Figures 3 and 6.

608 c) The OR in WT needle complexes assembles into a lower 16-mer, and an upper 15-mer
 609 ring complex (C16/C15) that are slightly tilted towards each other. The 16th protomer of
 610 InvG in the lower ring is highlighted in green (C1 reconstruction). The connecting
 611 densities at the furthest distance at the constriction are missing on one side (arrow). Cross
 612 section of the upper and lower OR at the indicated positions.

613 d) Western blot detection of a faster migrating InvG band in isolated WT needle
 614 complexes using a polyclonal antibody raised against the N0-N1 domains of InvG. The
 615 band is not detected in ΔEA mutant complexes. Other needle complex components are
 616 detected using an anti needle complex antibody.

617 e) Mild and selective needle complex disassembly. Class averages of vitrified samples
 618 prior and after ethanol treatment. In the absence of the IR, the lower OR does not
 619 maintain a ring conformation, whereas the upper OR stays intact. (avg (average) and
 620 stdev (standard deviation of class average). See also Supplementary Figures 3 and 6.

621 f) g) The C-terminus of InvG spans two neighboring InvG subunits and is critical for
 622 injectosome stability. Ribbon diagram of the entire 16/15-mer OR and zoomed in view in
 623 surface view. A single InvG (orange) interacts with two consecutive InvG protomers
 624 (G_{n+1} , G_{n+2} light grey and dark grey, respectively). Positions of truncations ($\Delta 6$ and $\Delta 10$)
 625 impacting complex assembly (g) are indicated with dashed line. Scale bar 50 nm. See
 626 also Supplementary Figures 3 and 6.

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631 **Figure 4: The structure of dead-end complexes**

632 a) Cryo electron microscopy structure of complexes isolated from a ΔEA strain. focused

633 reconstruction of the IR/OR (lower) in C1 revealed a 23:15 symmetry of IR:OR. Lower:

634 ribbon diagram of the IR/OR interaction. PrgH protomers showing the same

635 conformation at the C-terminal tails are indicated with the same colors. Zoomed view:

636 three protomers of PrgH with different conformations (H1, H2, H3), two protomers of

637 InvG (G1, G2). The symmetry break occurs at the position indicated with an arrow.

638 b) Three conformations of PrgH ('A', 'B', 'C') differ at the C-terminal tail of PrgH in WT

639 and in Δ EA complexes, respectively. Conformation 'A' binds to the outside of InvG,
640 whereas 'B' and 'C' intercalates between the N-terminal domains of InvG. In WT the 3
641 conformations are arranged symmetrically (24 protomers: 8x each conformation),
642 whereas in Δ EA complexes 7x conformation 'A', 7x 'B', and 9x 'C' are arranged within
643 the 23-mer PrgH ring.

644 Overall, the intercalating conformations 'B' and 'C' (362-392) have the same structural
645 fold but differ in their lateral position relative to the main structure of PrgH.

646

647

Figure 5: The PQR complex, PrgJ and PrgI form an intricate secretion path for the substrate

a) Distinct class averages of vitrified samples isolated from a WT resemble the transition from a basal body to the fully assembled needle complex. The internally growing

653 filament pushes towards the septum. In assembled needle complexes, the septum
654 underwent a conformational change to allow extra-basal polymerisation of the needle
655 filament.

656 b) Structure of the entire internal sub-structure. Six PrgJ and the initial five PrgI
657 protomers serve as adaptors from pseudo-helical to a true-helical symmetry of the needle
658 filament. Left: Overview of the EA to needle filament structure, shown as a surface side
659 view with EA components in white, PrgJ subunits in blue ribbons representation and
660 interlocked PrgIs in yellow (surface). Three cross-sections show the packing of PrgJ
661 connecting to the EA ('1'), PrgI adaptors (yellow), and the helical PrgI filament stabilized
662 by interactions (orange) to the surrounding upper InvG ring. (InvG ring not shown in side
663 view). PrgJ forms two-legged helix-turn-helix structures that serve as pillars to support
664 the filament (gray). (2) The helical arrangement of PrgJ is adapted by a single round of
665 five PrgI protomers (yellow), with a distorted N-terminal region (I*) similar to the
666 distortion observed at one N-terminal helical leg of a single PrgJ (J*) protomer. (3) The
667 now purely symmetrical filament extends upwards within the OR InvG N-terminal
668 secretin domain. Interacting residues of PrgI (N-termini) and InvG protomers are colored
669 in orange. All proteins are shown in cartoon representation.

670 c) Substrate path through the EA, PrgJ, and the PrgI filament. The EA confines the
671 substrate path that continues at the level of SpaP into a 12-16 Å wide channel.

672

682 transported by the T3SS and subsequently grow a filamentous sub-structure attached to
 683 the export apparatus. Transport of PrgJ and PrgI is dependent on the recruitment of the
 684 transiently interacting sorting platform harboring, among other components the T3SS-
 685 specific ATPase. During the growth of the inner PrgI filament, the closed septum in basal
 686 bodies undergoes a conformational change to allow extra-basal growth of the needle
 687 filament to continue. (IM – inner membrane; OR – outer membrane). SpaP – blue, SpaR
 688 – yellow, SpaQ – turquoise, SpaS – magenta, PrgK – grey, PrgH – orange, InvG – yellow,
 689 PrgJ – pink, PrgI – light grey)

690

Supplementary Methods

Title:

Structural control for the coordinated assembly into functional pathogenic type-3 secretion systems

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38

39 **Methods**

40 **Generation of knockout strains**

41 The InvG knockout was created following the protocol of¹. The overlapping sites on the
 42 knockout cassette were designed to substitute base pairs 8 to 1446 of the *invG* gene
 43 (*Salmonella enterica* serovar Typhimurium str. LT2, AE006468.2, bps 3,041,604 to
 44 3,043,292) to chloramphenicol resistance gene. The last 246 bps of *invG* were left intact
 45 to preserve the ribosomal binding site (RBS) for the next gene in the *inv* operon, thus
 46 avoiding polar effects of the deletion. The modified genomic region was subsequently
 47 transduced to a clean background strain (SB905)² by the *Salmonella*-specific phage P22.
 48 To remove the antibiotic resistance, the strain was transformed with the thermosensitive
 49 plasmid pCP20, containing the yeast *flp* recombinase gene, which removed the antibiotic
 50 resistance cassette via flanking FRT sites. The strain was cured from the plasmid by
 51 overnight incubation at 42°C. Knock-out strains for the entire export apparatus
 52 ($\Delta spaPQRS$, $\Delta invA$) and the individual export apparatus genes ($\Delta spaP$, $\Delta spaQ$, $\Delta spaR$,
 53 $\Delta spaS$, $\Delta invA$) have been described previously³.

54 Bacterial strains and phages used in this study are summarized in Supplementary Table 6.

55

56 **Complementation plasmids**

57 The $\Delta invG$ *Salmonella* strain (SB908) was complemented with WHS008 generated by
 58 replacing the PrgH sequence in the plasmid WHS006⁴. Additionally, a stop codon was
 59 introduced to avoid expression of the polyhistidine-tag.

C-terminal deletion variants of the complementation plasmids WHS006 (for $\Delta prgH$ complementation) and WHS008 (for $\Delta invG$ complementation) were generated by an inverse site-directed mutagenesis protocol utilizing and checked by Sanger sequencing. The plasmids were electroporated into $\Delta prgH$ or $\Delta invG$ *Salmonella* strains carrying the additional plasmid pSB1418, carrying *hila* gene, the main transcriptional regulator of SPI-1⁵ controlled by the arabinose promoter.

Plasmids used in this study are summarized in Supplementary Table 6.

Secretion assay

Secretion assays were conducted as previously described⁶ with minor modifications. Briefly, starter cultures were grown overnight at 37 °C in LB medium supplemented with 0.3 M NaCl and under antibiotic selection. Cultures were diluted 1:10 in 50 ml of the same medium without antibiotics. The expression of *hila* was induced by addition of 0.012% (w/v) L-arabinose for another growth period of 5 h to allow for the assembly of functional injectisomes and expression of effector proteins. Afterwards, the cell density was adjusted with LB media to an OD₆₀₀ of 1.0 and the samples were centrifuged (6000 g for 15 min) to pellet the cells. Supernatants were collected and filtered with a 0.22 µM syringe filter to remove any residual cells. Pellets were resuspended in 1x PBS (phosphate buffered saline). Both supernatants and cell pellets were then immunoblotted with primary polyclonal rabbit antibodies raised against proteins of interest and secondary anti-rabbit HRP (horse radish peroxidase) conjugated antibodies (Qiagen).

Antibodies used in this study are summarized in Supplementary Table 6.

Needle complex purification

Preparation of the needle complexes was performed as described previously^{6,7}. For assessing InvG and PrgH mutant/deletion versions, the protocol was scaled down by a factor of 4.

For selective needle complex disassembly, purified complexes were incubated with 20% v/v EtOH for 5 min at ambient temperature. The mixture was diluted with an equal volume of FR3 buffer (10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM EDTA., 0.1% LDAO) and loaded onto a Superose 6 10/300 GL column equilibrated with FR3 buffer at 4°C. Fractions containing selectively disassembled needle complexes were pooled together, and analyzed by negative staining transmission electron microscopy. Optionally, samples were concentrated using centrifugal concentrators with a 30 kDa molecular weight cutoff.

Co-variation/Evolutionary coupling analysis

Co-variation between pairs of T3SS proteins was assessed using the EVCouplings complex pipeline⁸ and RaptorX⁹. For EVCouplings the results were filtered to have a coupling score above 0.05, and probability score above 0.8; results with discrete EVC ratio were not considered. For RaptorX results the probability cutoff was 0.5. All results are summarized in Supplementary Table 2.

102

103 **Negative-stain electron microscopy**

104 Carbon coated copper grids (400mesh) were glow discharged (Bal-Tec, SCD 005) for 40
105 seconds at 20 mA. Five μ l of sample was applied to the grid and incubated for 30
106 seconds. The sample was washed off with 5 μ l of staining solution (2% phospho-tungsten
107 acetate, adjusted to pH 7.0 with NaOH) and stained with 5 μ l of the staining solution for
108 20 sec. Micrographs were obtained at Morgagni 268D microscope (FEI) equipped with
109 an 11 megapixel CCD camera (Olympus-SIS, Morada) or at a Talos L120C (FEI) with a
110 4K Ceta CEMOS camera.

111

112 **Measurement and statistical analysis of inner ring diameters**

113 Pixel intensity values corresponding to the inner ring were extracted from class average
114 images and averaged along the vertical Y-axis for each class. IR diameters were
115 calculated as the distance between the two minima of the averaged profile (Figure 1c,d,
116 Supplementary Figure 1). Resulting diameter values were tested for normality using
117 either D'Agostino-Pearson omnibus K2 test (for n=8 and above) or Shapiro-Wilk test (for
118 n <8) with 95% confidence interval. Levene's test with 95% confidence interval was used
119 to assess the equality of variance. Unpaired t-test with 95% confidence interval was used
120 to compare diameter measurements of mutants to the WT diameter. p-values are provided
121 in Supplementary Table 1. Statistical analysis was performed in Python 2.7 using
122 modules scipy.stats¹⁰ and pandas¹¹.

123

124 **Sample vitrification and cryo electron microscopy**

125 For cryo electron microscopy, samples were vitrified on Quantifoil 1.2/1.3, 2/1, and 2/2
126 with either an additional home-made layer of amorphous carbon ($<1.6\text{nm}$) or graphene
127 oxide¹². Briefly, 4 μl of sample was applied onto glow-discharged grids and allowed to
128 disperse for 0.5-2min. The grids were blotted for 4-7 s set at 100% humidity and plunge-
129 frozen in liquid propane/ethane mixture cooled with liquid nitrogen to about minus 180-
130 190 °C, by using a Vitrobot Mark V.

131 Vitrified specimens were imaged on a FEI Titan Krios operating at 300 kV and equipped
132 with a field emission gun (XFEG) and a Gatan Bioquantum energy filter. Movies
133 consisting of 25 frames were automatically recorded using FEI EPU software and the K2
134 Summit camera in counting mode at a nominal magnification of 130kx, corresponding to
135 1.09 Å per physical pixel. For individual frames, an electron dose of 1.1-1.26 $\text{e}^-/\text{Å}^2$ was
136 used, corresponding to a cumulative electron dose of 27.5-31.5 $\text{e}^-/\text{Å}^2$ equally distributed
137 over 5 sec movie. Movies were recorded at -0.8 - 3.5 μm defocus. Samples for diameter
138 measurements were recorded with LEGION¹³ on a FEI Polara (300kV) equipped with
139 field emission gun (FEG) and a Gatan CCD Camera (UHS 4000). Total electron dose was
140 35 $\text{e}^-/\text{Å}^2$. The information on the datasets is summarized in Supplementary Table 3.

141

142 **Single-particle reconstruction**

143 Single particle reconstructions were performed using Relion3¹⁴. Movies were motion-

corrected and dose-weighted and the CTF of the resulting micrographs was determined using CTFFIND4¹⁵. Particles were picked from the motion-corrected micrographs using Cryolo¹⁶ trained with a sub-set of manually picked particles (4-fold binned. Particles were extracted using a boxsize of 432 pixels and subsequently binned four times for several rounds of 2D classifications. A cleaned and unbinned dataset was obtained by re-extraction and aligned to a rotationally averaged structure. Focused refinements with and without applying symmetry were performed to the individual sub-structures (Supplementary Table 4) using respective 3D masks. After converged refinements, per-particle CTF and Bayesian polishing was used to generate new data sets for another round of focused refinements. Overall gold-standard resolution (Fourier shell correlation (FSC) = 0.143) and local resolution were calculated with Relion3. (Data sets from WT needle complex recorded over amorphous carbon and graphene oxide were processed separately and combined into a single data set after Bayesian polishing).

Helical reconstruction of the outer needle filament

Helical reconstruction was performed in Relion3¹⁷ on the carbon dataset used for high-resolution refinement of the needle complex. The box center was moved from its original position at the basal body to the approximate middle of the filament by recalculating the particle coordinates according to the formulae:

$$X' = X - \Delta X * \cos(\psi) - \Delta Y * \sin(\psi)$$

$$Y' = Y + \Delta X * \sin(\psi) - \Delta Y * \cos(\psi)$$

Where X, Y are the initial particle coordinates (rlnCoordinateX and rlnCoordinateY fields in *.star file), ΔX and ΔY are the shifts of the particle as measured on a 2D class average and ψ is the in-plane rotation angle (rlnAnglePsi field in *.star file).

Re-extracted particles were subjected to 2D classification. Classes where the helicity of the filament was most pronounced were used to convert box center coordinates to helix start and end coordinates using the formula above. Calculations of new coordinates were done with Python 2.7 and modules pandas and numpy^{11,18}. Segments were extracted from micrographs into 300 pixel boxes with 28 pixel inter-box distance (defined by helical rise of 4.2 Å and 6 asymmetric units). In total 303 155 segments were extracted. The dataset was classified in 2D with tube diameter set to 95 Å and fine angular sampling. Classes that showed no evidence of overfitting and little or no extra density from the IR were selected for 3D auto-refinement procedure (total 133516 segments). Tube diameter was set to 95 Å, and the initial values for helical twist and rise were 64° and 4.2 Å (as determined in¹⁹). Helical parameter search range was $\pm 20\%$, and a soft-edged cylinder with diameter 95 Å was used as an initial model. The resulting model was then subjected to CTF refinement and Bayesian polishing. Where applicable the initial models were low-pass filtered to 25 Å to keep the helical lattice intact while removing any high-resolution components. The map, filtered according to its local resolution in Relion, was symmetrized in real space using the refined values of helical twist and rise to generate the final map.

Building, refinement and validation of needle complex models

Templates and initial model generation

Existing models of PrgH (PDB: 3GR1) and PrgK (PDB: 3GR5), and InvG (4G08, G34-I173; lower OR) were preliminary placed into the respective EM maps (IR: WT 24-mer, ΔEA 23-mer; lower OR: WT 16mer, ΔEA 15mer; Supplementary Table 4) utilizing UCSF Chimera's `fitmap` command²⁰. The models were further refined using Rosetta²¹ controlled via StarMap 1.0 (manuscript in preparation).

The upper OR was modelled into the 15mer symmetrized EM map by building InvG (R177-G557) *ab initio* with Coot²², starting with well defined secondary structure elements. The growing model was iteratively refined using Rosetta (controlled by Starmap), followed by several rounds of model extension and manual improvement in Coot.

Models for the upper and lower OR were connected in Coot based on densities obtained through focused refinements of the neck region.

Homology models for SpaP, SpaQ, SpaR, SpaS were generated with SWISS-MODEL²³ based on FliP, FliQ, FliR, and FliS from *Vibrio mimicus* (personal communication, S. Lea); PrgJ was modelled using Phyre2²⁴. PrgI (PDB 2LPZ) and models for SpaP, SpaQ, SpaR, SpaS, and PrgJ were preliminary placed into the respective EM map, extended if necessary, and refined using Rosetta (Starmap) and Coot.

208 *Model refinement and validation*

209 All resulting models were manually extended and refined using Coot, and subsequently
210 subjected to another round of refinement using Rosetta. The quality of the individual
211 models were analyzed using the MolProbity server ²⁵ and parts of the models further
212 refined if necessary.

213 Overall map/model FSCs as well as Z-scores ²⁶ were calculated with Starmap to assess
214 the quality of the models. Z-scores were colored for each model using Chimera ²⁰.

215

216 **Visualization, analysis and deposition**

217 UCSF Chimera ²⁰ and ChimeraX ²⁷ were used for molecular visualization. Analysis of
218 interaction surfaces between inner ring components PrgH and PrgK in the WT and ΔEA
219 T3SS needle complex were performed with the PDBePISA server ²⁸. Angles in InvG were
220 measured with MB-Ruler (MB-Software solutions, version 5.3). Plots were made with
221 matplotlib ²⁹.

222 EM maps are deposited at the EMDB (<https://www.emdataresource.org>). Coordinates of
223 models are deposited at the ePDB database. Supplementary Table 4 summarizes the
224 depositions submitted in this study.

225

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295

296 **Supplementary figures**

298 **Supplementary Figure 1: T3SS-complexes from WT and mutant strains display**
 299 **different IR diameters**

300 Two-dimensional class averages from vitrified complexes (WT, ΔEA , $\Delta invG$, $\Delta invA$,
 301 $\Delta spaS$, $\Delta spaP$, $\Delta spaQ$, $\Delta spaR$) and density profile along IR. Diameters were measured as
 302 the distance of the two lowest minima along the density profile (dashed lines: standard
 303 deviation) and summarized in Supplementary Table 1.

304

305

306 **Supplementary Figure 2: The PQR complex adopts a pseudo-helical assembly.**

307 a) Surface views from the PQR complex at different orientations (top, side, bottom).

308 Proteins are colored according to their identity (SpaP: blue; SpaR: yellow, SpaQ: grey)

309 b) Surface views of the pseudo-helical packing of the individual SpaP-group, SpaR, and

310 SpaQ-group. SpaQ resembles the bottom of the PQR complex, whereas the SpaP group is

311 arranged at the top-rim of the complex

312

Supplementary Figure 3: C-terminal truncations of PrgH or InvG impair T3SS function.

a) Secretion assays for C-terminal PrgH truncations. Deletions of four amino acids do not, and more than 10 amino acids render the T3SS non-functional. Left/top: Detection of needle complex components (NC) in cell pellets, Top/right: Detection of InvG in PrgH-truncated cells; Bottom/left: Detection of late T3SS-substrate SptP in cell pellets. Bottom/right: Detection of secreted late T3SS-substrate SptP in cell culture supernatants. (WT: SB905, SB906: $\Delta prgH$, WHS06: $\Delta prgH$ (SB906), complemented with plasmid-borne WT *prgH*), $\Delta 4$ - $\Delta 39$: $\Delta prgH$ (SB906) complemented with plasmid-borne C-terminal PrgH deletions; anti-NC (anti needle complex antibody).

b) Secretion assay for C-terminal InvG truncations. Deletions of six amino acids do not, and more than 10 amino acids render T3SS non-functional. Left/top: Detection of needle complex components (NC) in cell pellets, Top/right: Detection of InvG and truncated InvG in cells; Bottom/left: Detection of late T3SS-substrate SptP in cell pellets. Bottom/right: Detection of secreted late T3SS-substrate SptP in cell culture supernatants. (WT: SB905, $\Delta invG$: SB908, $\Delta InvG$ + comp.: SB908 complemented with plasmid-borne WT *invG*, $\Delta 3$ - $\Delta 45$: $\Delta invG$ complemented with plasmid-borne C-terminal InvG deletions; anti-NC (anti needle complex antibody).

333

334 **Supplementary Figure 4: The dead-end injectisome assembly**

335 a) PrgH and PrgK engage in a similar intertwined packing to form the IR in WT and Δ EA
 336 complexes. PrgH (blue, one protomer) is oriented perpendicular relative to the IR,
 337 whereas PrgK (yellow, one protomer) binds to the PrgH-ring at an angle between 24
 338 (Δ EA) and 27°. (WT). In this view, the upper PrgK domain binds to PrgH shown in blue;
 339 the lower PrgK domain binds to the neighboring PrgH protomer (+1).

340 b) The PQR complex does not fit into a 23-mer complex, and provides a structural
 341 template for the WT 24-mer IR. Inner circle defined by the 24-mer WT complex
 342 (continuous line) and the 23-mer Δ EA complex (dashed line). Surface view through IR,
 343 SpaP (blue), SpaR (yellow), SpaQ (dark grey), SpaS (green). The surface of PrgK is
 344 colored according to hydrophobicity (see also Figure 2), and the section depicted in light
 345 grey.

346

Supplementary Figure 5: Grid support type affects needle complex particle distribution in cryo electron microscopy

- a) Exemplary data for needle complexes vitrified on grids containing a graphene oxide support layer. Top: Cryo electron micrograph (5-fold binned and 25 Å low-pass filtered in Relion), scale bar 50 nm. Middle: Histograms of Euler angle distribution (Rot and Tilt) of asymmetric 3D refinement created using the `plot_indivEuler_histogram_fromStarFile.py` (Michael A. Cianfrocco, U. Michigan) and histogram of Euler angle distribution (Tilt).
- b) Same as in (a), yet for continuous carbon grids.
- c) Angular distribution of C1 reconstruction (job373) of the sub-dataset focusing on the WT export apparatus complex. Distribution shown from the side and rotated 90° along the x-axis. Lighter colors (magenta) indicate a higher number of particles found with the respective tilt angle.

360

361 **Supplementary Figure 6: Stability of T3SS needle complexes is impaired by C-**
 362 **terminal PrgH or InvG truncations.**

363 Representative images of isolated complexes from WT, Δ *prgH*, Δ *invG* and plasmid-

364 complemented strains. Plasmid-borne truncated versions of PrgH and InvG are indicated.
 365 (Middle) PrgH C-terminal deletions – $\Delta 4$, 10, 16, 21 and 25; (Right) InvG C-terminal
 366 deletions – $\Delta 3$, 6, 10, 14 and 24; Scale bar 50 nm.

368 **Supplementary Figure 7: Export apparatus knock-out basal-bodies are unstable in**
 369 **negative-stain EM**

370 Isolated complexes from a strain lacking the entire export apparatus proteins (Δ EA:
 371 $\Delta(\textit{spaP}, \textit{spaQ}, \textit{spaR}, \textit{spaS}, \textit{invA})$), and the individual genes for *spaP*, *spaQ*, *spaR* are
 372 sensitive to structural disruption upon negative stain electron microscopy, likely through
 373 shear forces during the staining procedure. However, the same samples withstand
 374 vitrification for cryo electron microscopy.

376 **Supplementary Figure 8: Summary for asymmetric reconstruction of WT export**
 377 **apparatus**

- 378 a) Schematic showing the location of the complex in inside the needle complex
- 379 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 380 (blue). The resolution at the respective cut-off is given.
- 381 c) Left: post-processed electron density map, colored by local resolution (Relion
- 382 implementation); right: ribbon view of the model colored by Rosetta Z-score

384 **Supplementary Figure 9: Summary for C8 reconstruction of WT IR and OR lower**
 385 **region**

- 386 a) Schematic showing the location of the complex in inside the needle complex
- 387 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
 388 (blue). The resolution at the respective cut-off is given.
- 389 c) Left: post-processed electron density map, colored by local resolution (Relion
 390 implementation); right: ribbon view of the model
- 391 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

393 **Supplementary Figure 10: Summary for asymmetric reconstruction of WT OR**

394 a) Schematic showing the location of the complex in inside the needle complex

395 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model

396 (blue). The resolution at the respective cut-off is given.

397 c) Left: post-processed electron density map, colored by local resolution (Relion

398 implementation); right: ribbon view of the model colored by Rosetta Z-score

400 **Supplementary Figure 11: Summary for C15 reconstruction of WT InvG upper**
 401 **region**

- 402 a) Schematic showing the location of the complex in inside the needle complex
- 403 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 404 (blue). The resolution at the respective cut-off is given.
- 405 c) Left: post-processed electron density map, colored by local resolution (Relion
- 406 implementation); right: ribbon view of the model
- 407 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

409 **Supplementary Figure 12: Summary for C16 reconstruction of WT InvG lower**
 410 **region**

- 411 a) Schematic showing the location of the complex in inside the needle complex
- 412 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 413 (blue). The resolution at the respective cut-off is given.
- 414 c) Left: post-processed electron density map, colored by local resolution (Relion
- 415 implementation); right: ribbon view of the model
- 416 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

418 Supplementary Figure 13: Summary for C24 reconstruction of WT IR

- 419 a) Schematic showing the location of the complex in inside the needle complex
- 420 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 421 (blue). The resolution at the respective cut-off is given.
- 422 c) Left: post-processed electron density map, colored by local resolution (Relion
- 423 implementation); right: ribbon view of the model
- 424 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

426 **Supplementary Figure 14: Summary for asymmetric reconstruction of WT inner**
 427 **filament part**

- 428 a) Schematic showing the location of the complex in inside the needle complex
- 429 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
 430 (blue). The resolution at the respective cut-off is given.
- 431 c) Left: post-processed electron density map, colored by local resolution (Relion
 432 implementation); right: ribbon view of the model colored by Rosetta Z-score

434 **Supplementary Figure 15: Summary for helical reconstruction of WT outer filament**
 435 **part**

- 436 a) Schematic showing the location of the complex in inside the needle complex
- 437 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 438 (blue). The resolution at the respective cut-off is given.
- 439 c) Left: post-processed electron density map, colored by local resolution (Relion
- 440 implementation); right: ribbon view of the model
- 441 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

443 **Supplementary Figure 16: Summary for asymmetric reconstruction of Δ EA export**

444 **IR and InvG lower region**

445 a) Schematic showing the location of the complex in inside the needle complex

446 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model

447 (blue). The resolution at the respective cut-off is given.

448 c) Left: post-processed electron density map, colored by local resolution (Relion

449 implementation); right: ribbon view of the model colored by Rosetta Z-score

451 **Supplementary Figure 17: Summary for C15 reconstruction of Δ EA InvG upper**
 452 **region**

- 453 a) Schematic showing the location of the complex in inside the needle complex
- 454 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 455 (blue). The resolution at the respective cut-off is given.
- 456 c) Left: post-processed electron density map, colored by local resolution (Relion
- 457 implementation); right: ribbon view of the model
- 458 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

460 **Supplementary Figure 18: Summary for C23 reconstruction of Δ EA IR**

- 461 a) Schematic showing the location of the complex in inside the needle complex
- 462 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
- 463 (blue). The resolution at the respective cut-off is given.
- 464 c) Left: post-processed electron density map, colored by local resolution (Relion
- 465 implementation); right: ribbon view of the model
- 466 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

468 **Supplementary Figure 19: Summary for C15 reconstruction of Δ EA InvG lower**
 469 **region**

- 470 a) Schematic showing the location of the complex in inside the needle complex
- 471 b) Fourier-shell correlation plots of the: map versus map (grey) and map versus model
 472 (blue). The resolution at the respective cut-off is given.
- 473 c) Left: post-processed electron density map, colored by local resolution (Relion
 474 implementation); right: ribbon view of the model
- 475 d) An asymmetric unit in ribbon view colored by Rosetta Z-score

Supplementary Table 1: Statistical analysis of IR diameters from 2D class averages

Sample	n	Diameter, pix	Normality test	Normality test p-value	Normal distribution	Levene test p-value	Equal variance to WT	t-test p-value	IR oligomericity
WT	8	135.88±0.99	D'Agostino-Pearson	0.03	No	-	-	-	24
Δ EA	8	132.88±1.55	D'Agostino-Pearson	0.59	Yes	0.25	Yes	0.0004	23
Δ spaP	8	134.13±0.83	D'Agostino-Pearson	0.56	Yes	1.00	Yes	0.0019	23
Δ spaQ	8	132.38±0.74	D'Agostino-Pearson	0.54	Yes	0.91	Yes	<0.0001	23
Δ spaR	8	132.38±0.92	D'Agostino-Pearson	0.73	Yes	0.84	Yes	<0.0001	23
Δ invG	8	137±1.51	D'Agostino-Pearson	0.21	Yes	0.46	Yes	0.1002	24
Δ invA	8	136.63±1.19	D'Agostino-Pearson	0.19	Yes	0.55	Yes	0.1918	24
Δ spaS (type 1)	9	135.22±1.64	D'Agostino-Pearson	0.01	No	0.25	Yes	0.3446	24
Δ spaS (type 2)	5	131.6±1.67	Shapiro-Wilk	0.31	Yes	0.18	Yes	0.0001	23

Diameter values are given as average $\pm$ standard deviation from n class averages

For all tests the confidence interval is 95%

Supplementary Table 2: Evolutionary couplings between T3SS proteins

Protein A	Position in A	Protein B	Position in B	EVC Ratio	EVC Coupling Score	EVC Coupling Score Probability	RaptorX Probability	Notes
InvG177		PrgH		discrete				
InvG177		PrgK		good				
PrgH		PrgK		discrete				
PrgI		SpaP		good				
PrgJ		PrgI		good				
PrgJ	88	SpaP	12	discrete	0.069921	0.80929057013		
SpaP		InvG177		good				
SpaP		PrgK		very good				
SpaP	160	PrgK	95				0.639075	
SpaP	160	PrgK	94				0.600327	
SpaP	157	PrgK	95				0.574201	
SpaQ		PrgK		very good				
SpaS		PrgK		very good				
SpaS		SpaP		very good				
SpaS	123	SpaQ	39	very good	0.228503	0.99999646681	0.81136	Found in both EVC and RaptorX
SpaS	117	SpaQ	38	very good	0.140312	0.95004729629	0.752302	Found in both EVC and RaptorX
SpaS	105	SpaQ	39	very good	0.129688	0.88339858147		
SpaS	135	SpaQ	39	very good	0.186191	0.99943133179		
SpaS	104	SpaQ	41	very good	0.15712	0.98894493295	0.690507	Found in both EVC and RaptorX
SpaS	117	SpaQ	39				0.881415	
SpaS	104	SpaQ	38				0.701592	
SpaS	119	SpaQ	39				0.626436	
SpaS	305	SpaQ	45				0.624521	
SpaS	122	SpaQ	39				0.60804	
SpaS	118	SpaQ	39				0.535562	
SpaS	126	SpaQ	39				0.531625	
SpaS	135	SpaQ	39				0.526511	
SpaS	86	SpaQ	54				0.522819	
SpaS	68	SpaQ	72				0.503434	
SpaS	149	SpaR	198	very good	0.278174	1	0.874956	Found in both EVC and RaptorX
SpaS	171	SpaR	186	very good	0.36968	1	0.916678	Found in both EVC and RaptorX
SpaS	171	SpaR	190	very good	0.134256	0.99950400538	0.729069	Found in both EVC and RaptorX
SpaS	179	SpaR	197	very good	0.171552	0.99999980529	0.915408	Found in both EVC and RaptorX
SpaS	179	SpaR	193	very good	0.103505	0.92697559046	0.69132	Found in both EVC and RaptorX
SpaS	182	SpaR	194	very good	0.1012	0.90099992619	0.775293	Found in both EVC and RaptorX
SpaS	163	SpaR	190	very good	0.130257	0.99897334881	0.666919	Found in both EVC and RaptorX
SpaS	175	SpaR	190	very good	0.323811	1	0.87269	Found in both EVC and RaptorX
SpaS	186	SpaR	201				0.93371	
SpaS	182	SpaR	197				0.88573	
SpaS	164	SpaR	187				0.876829	
SpaS	168	SpaR	183				0.842796	
SpaS	182	SpaR	198				0.833507	
SpaS	168	SpaR	182				0.831055	
SpaS	183	SpaR	197				0.827168	
SpaS	160	SpaR	190				0.772297	
SpaS	163	SpaR	187				0.766828	
SpaS	178	SpaR	194				0.753234	
SpaS	183	SpaR	201				0.747192	
SpaS	160	SpaR	191				0.73799	
SpaS	179	SpaR	194				0.711606	
SpaS	190	SpaR	205				0.708529	
SpaS	153	SpaR	194				0.704504	
SpaS	168	SpaR	186				0.69983	
SpaS	164	SpaR	183				0.698627	
SpaS	166	SpaR	183				0.691212	
SpaS	160	SpaR	187				0.679686	
SpaS	160	SpaR	194				0.675681	
SpaS	186	SpaR	198				0.666855	
SpaS	145	SpaR	202				0.654103	
SpaS	167	SpaR	183				0.639143	
SpaS	164	SpaR	233				0.623913	
SpaS	186	SpaR	202				0.608171	

SpaS	168	SpaR	179				0.606976	
SpaS	161	SpaR	233				0.600034	
SpaS	164	SpaR	237				0.599347	
SpaS	175	SpaR	193				0.591811	
SpaS	165	SpaR	183				0.589167	
SpaS	153	SpaR	198				0.582776	
SpaS	141	SpaR	211				0.57742	
SpaS	157	SpaR	229				0.567868	
SpaS	182	SpaR	201				0.566108	
SpaS	190	SpaR	201				0.565288	
SpaS	141	SpaR	206				0.553542	
SpaS	164	SpaR	234				0.542084	
SpaS	161	SpaR	230				0.541381	
SpaS	171	SpaR	183				0.539712	
SpaS	164	SpaR	184				0.538042	
SpaS	149	SpaR	195				0.537265	
SpaS	187	SpaR	201				0.533428	
SpaS	164	SpaR	190				0.523606	
SpaS	160	SpaR	230				0.520204	
SpaS	138	SpaR	207				0.514231	
SpaS	152	SpaR	198				0.511071	
SpaS	141	SpaR	207				0.5069	

Empty fields mean no data available

EVC ratio characterizes the alignment quality, but this does not guarantee any intermol couplings to be found

RaptorX does not output residue number, this has to be checked manually

InvG177 only first 177 residues of InvG were analysed

Supplementary Table 3: Cryo-EM data collection summary

Sample		WT	WT	EA-KO
	Buffer	10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM EDTA., 0.1% LDAO		
	Vitrification			
	Grid	graphene oxide	carbon	carbon
Data collection	Magnification, nominal	130x	130x	130x
	Energy filter slit, eV	not used	10-15	10-15
	Defocus range, μm	0.7-5.1	0.3-5.2	0.4-5.1
	Voltage, kV	300	300	300
	Microscope	Titan Krios	Titan Krios	Titan Krios
	Camera	Gatan K2	Gatan K2	Gatan K2
	Frame exposure time, s	0.2	0.2	0.2
	Dose per frame	1.24	1.26	1.1
	Number of movie frames	25	25	25
	Total electron dose, $\text{e}^-/\text{\AA}^2$	31	31.5	27.5
	Pixel size, $\text{\AA}$	1.09	1.09	1.09
	Number of micrographs (for motioncorrection)	9352	10433	8190

Supplementary Table 4: Summary of maps and models

[illegible]

Supplementary Table 5: RMSD between SpaP and SpaQ conformations in EA
SpaP

	P1	P2	P3	P4	P5
P1	-				
P2	1.739	-			
P3	1.489	1.478	-		
P4	2.871	3.446	3.104	-	
P5	3.206	2.79	3.148	3.766	-

SpaQ

	Q1	Q2	Q3	Q4
Q1	-			
Q2	1.726	-		
Q3	2.165	1.32	-	
Q4	2.418	2.265	2.326	-

All values are in Å, calculated in UCSF Chimera after a structure/sequence alignment with MatchMaker

Table 6: Strains, plasmids and antibodies used in this study

Salmonella strains	Description	Parent strain	Reference*
SB905	Wild type strain, flagellar KO ...	SJW2941	2
SB906	Deletion of <i>prgH</i> gene	SB905	gift from Galán lab
SB908	Deletion of <i>invG</i> gene	SB905	This work
SB1171	Deletion of <i>invG</i> gene	SB905	gift from Galán lab
WHS06	SB906 complemented with WHS006	SB905	gift from Galán lab
WHS08	SB908 complemented with WHS008	SB905	This work
Plasmids	Description	Parent plasmid	Reference
WHS006	Complementation plasmid with <i>prgH</i> gene	N/A	gift from Galán lab
WHS008	Complementation plasmid with <i>invG</i> gene	WHS006	This work
pCP20	Encodes yeast Flp recombinase	N/A	30
pSB1418	Encodes <i>hliA</i> gene - overexpression of the SPI-I T3SS	N/A	31
pACYC-SptP3-GFP	Encodes trapped substrate with GFP	pACYC	6
Phage	Description		
P22	Salmonella specific phage - transfer of genetically modified area to a clean background		
Antibody	Description	Specific target	Reference
anti-NC	rabbit anti NC polyclonal antibody	Needle complex	6
anti-InvG	rabbit anti InvG polyclonal antibody	InvG domains N0-N1	Medical University of Vienna, Department of Medical Biochemistry, Prof. Hermann
anti-SptP	rabbit anti SptP polyclonal antibody	SptP amino acids 1-295	6

* reference numbering as in Materials and Methods

Supplementary Table 7: T3SS protein naming

Protein name	Unified nomenclature	UniProt ID	Flagellar homologue
InvA	SctV	P0A1I3	FlhA
InvG	SctC	P35672	-
PrgH	SctD	P41783	FliG
PrgI	SctF	P41784	-
PrgJ	SctI	P41785	-
PrgK	SctJ	P41786	FliF
SpaP	SctR	P40700	FliP
SpaQ	SctS	P0A1L7	FliQ
SpaR	SctT	P40701	FliR
SpaS	SctU	P40702	FlhB