

1 **Visualization of the type III secretion mediated *Salmonella*–host cell interface using cryo-
2 electron tomography**

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18 **ABSTRACT**

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20 **Many important gram-negative bacterial pathogens use highly sophisticated type III secretion**
21 **systems (T3SSs) to establish complex host-pathogen interactions. Bacterial-host cell contact**
22 **triggers the activation of the T3SS and the subsequent insertion of a translocon pore into the**
23 **target cell membrane, which serves as a conduit for the passage of effector proteins. Therefore**
24 **the initial interaction between T3SS-bearing bacteria and host cells is the critical step in the**
25 **deployment of the protein secretion machine, yet this process remains poorly understood. Here,**
26 **we use high-throughput cryo-electron tomography (cryo-ET) to visualize the T3SS-mediated**
27 ***Salmonella*-host cell interface. Our analysis reveals the intact translocon at an unprecedented**
28 **level of resolution, its deployment in the host cell membrane, and the establishment of an**
29 **intimate association between the bacteria and the target cells, which is essential for effector**
30 **translocation. Our studies provide critical data supporting the long postulated direct injection**
31 **model for effector translocation.**

32

33 **INTRODUCTION**

34

35 Type III secretion systems (T3SSs) are widely utilized by many pathogenic or symbiotic Gram-
36 negative bacteria to directly inject bacterially encoded effector proteins into eukaryotic host cells
37 (Deng et al, 2017; Galán et al, 2014; Notti & Stebbins, 2016). The central element of the T3SS is the
38 injectisome, a multiple-protein machine that mediates the selection and translocation of the effectors
39 destined to travel this delivery pathway. The injectisome is highly conserved, both structurally and
40 functionally, among different bacterial species including important pathogens such as *Salmonella*,
41 *Yersinia*, *Shigella*, *Pseudomonas* and *Chlamydia* species. It consists of defined substructures such as
42 the needle complex, the export apparatus, and the cytoplasmic sorting platform (Galán et al, 2014; Hu
43 et al, 2017; Loquet et al, 2012; Schraadt & Marlovits, 2011; Worrall et al, 2016). The needle complex is
44 composed of a membrane-anchored base, a protruding needle filament, and a tip complex at the
45 distal end of the needle (Kubori et al, 1998; Schraadt et al, 2010; Schraadt & Marlovits, 2011; Worrall et
46 al, 2016). The export apparatus, which is formed by several inner membrane proteins, functions as
47 the conduit for substrate translocation across the bacterial inner membrane (Dietsche et al, 2016). The
48 sorting platform is a large cytoplasmic multiple-protein complex that orderly selects and delivers the
49 substrates to the export apparatus (Lara-Tejero et al, 2011).

50 In many bacterial species the activity of these protein injection machines is stimulated upon
51 contact with the target eukaryotic cell plasma membrane, a process thought to be mediated by the tip
52 complex (Barta et al, 2012; Blocker et al, 2008; Deane. JE et al, 2006; Ménard et al, 1994; Zierler & Galán,
53 1995). Host cell contact triggers a cascade of poorly understood events that lead to the deployment of
54 the protein translocases onto the host cell plasma membrane to form a protein channel in the host cell
55 membrane that mediates the passage of the effector proteins. In the case of the *Salmonella enterica*
56 serovar Typhimurium (*S. Typhimurium*) T3SS encoded within its pathogenicity island 1, the protein
57 translocases are SipB and SipC , which through a process that requires the tip protein SipD, are
58 inserted in the host-cell plasma membrane to form the translocon channel (Collazo & Galán, 1997).

59 Deployment of the translocon also results in the intimate association of the bacteria and the host cell,
60 which is orchestrated by the protein translocases themselves (Lara-Tejero & Galán, 2009; Misselwitz
61 et al, 2011). Despite the critical role of the translocases in intimate attachment and effector
62 translocation, little is known about their structural organization when deployed in the host cell
63 membrane, and previous attempts to visualize them did not provide distinct structural details. This
64 paucity of information is due at least in part to the intrinsic difficulties of imaging bacterial/host cell
65 interactions at high resolution. Here, we used bacterial minicells and cultured mammalian cells
66 combined with high-throughput cryo-ET to study the initial interaction between *S. Typhimurium* and
67 host cells. This experimental system allowed the visualization of the intact translocon deployed in the
68 host cell plasma membrane, in contact with the tip-complex of the T3SS injectisome, at
69 unprecedented resolution. This study provides new insights into the initial events of the T3SS-
70 mediated bacteria-host cell interactions and highlights the potential of cryo-ET as a valuable tool for
71 investigating the host cell-pathogen interface.

72

73 RESULTS

74

75 ***in situ* structures of the T3SS injectisome in the presence or absence of protein translocases**

76 An intrinsic property of many T3SSs is that their activity is stimulated by contact with the target
77 host cell plasma membrane (Ménard et al, 1994; Zierler & Galán, 1995). This interaction results not
78 only in the stimulation of secretion but also in the deployment of the protein translocases in the host cell
79 membrane, a poorly understood process that is orchestrated by the tip complex of the injectisome's
80 needle filament. In the case of the *S. Typhimurium* SPI-1 T3SS the tip complex is thought to be
81 composed of a single protein, SipD, which organizes as a pentamer at the tip of the needle filament
82 (Rathinavelan et al, 2014). However, it has been previously proposed that in *Shigella* spp., in addition
83 to IpaD, a homolog of SipD, the tip complex also contains IpaB, a homolog of SipB (Cheung et al, 2015).
84 To get insight into the structural organization of the tip complex prior to bacterial contact with cultured

85 cells, we compared the *in situ* structures of fully assembled injectisomes from minicells obtained from
86 wild-type, $\Delta sipB$, and $\Delta sipD$ *S. Typhimurium* strains (Fig. 1a-d, Extended Data Table 1). We found that
87 injectisomes from wild-type or the $\Delta sipB$ strains were indistinguishable from one another. In contrast,
88 injectisomes from a $\Delta sipD$ strains exhibited a shorter needle (~45 nm) in comparison to the needle
89 filaments of injectisomes from the wild-type or $\Delta sipB$ strains (~50 nm). These observations suggest that
90 SipD is the only structural component of the tip complex (Fig. 1e). To further explore this hypothesis,
91 we examined by cryo-ET the injectisomes of minicells obtained from *S. Typhimurium* strains expressing
92 FLAG-epitope-tagged versions of SipB, SipC, and SipD, after labeling with anti-FLAG antibodies (Fig.
93 1f-h). Only injectisomes from minicells obtained from the strain expressing SipD-FLAG showed the
94 antibodies bound to the needle tip (Extended Data Table 2). This observation is consistent with the
95 notion that, prior to cell contact, SipD is the main, and most likely only component of the tip-complex
96 (Lara-Tejero & Galán, 2009).

97

98 **High-resolution imaging of the T3SS mediated *Salmonella*-host cell interface**

99 It is well established that effector translocation through the T3SS requires an intimate
100 association between the bacteria and the host cell (Grosdent et al, 2002). It has also been previously
101 demonstrated that such intimate attachment requires an intact type III secretion machine, and in
102 particular, the protein translocases, which most likely mediate such bacteria/host cell interaction
103 (Lara-Tejero & Galán, 2009). Despite its central role in effector translocation, however, very little is
104 known about the architecture of this specialized host/bacteria interface. This is largely because of the
105 lack of amenable experimental approaches that would allow a detail view of this interface. Cryo-ET is
106 uniquely suited to examine host/pathogen interactions at high resolution. However, sample thickness
107 limits the utility of this approach. To get around this limitation we used bacterial minicells as a
108 surrogate for whole bacteria since it has been previously shown that they are capable of assembling
109 functional T3SS injectisomes that can deliver de novo synthesized T3SS substrates into cultured cells
110 (Carleton et al, 2013). However, minicells are inefficient at triggering membrane ruffling, actin filament

111 remodeling, and bacterial internalization due to inefficient partitioning of the effector proteins that
112 trigger these responses. Consequently, while minicells are proficient at establishing a T3SS-mediated
113 intimate association with cultured epithelial cells, they are inefficient at triggering their own
114 internalization thus remaining firmly attached on the cell surface. These features make them ideally
115 suited for high-resolution cryo-ET imaging. Therefore, we applied bacterial minicells obtained from
116 wild-type *S. Typhimurium* onto cultured epithelial cells grown on cryo-EM grids. We found that the
117 periphery of adherent cells is sufficiently thin (< 500 nm) for high-resolution imaging (Extended Data
118 Fig. 1). We readily observed T3SS injectisomes at the interface between minicells and the plasma
119 membrane of cultured epithelial cells (Fig. 2a-b). We found that in the presence of the injectisomes,
120 the spacing between the surface of the *S. Typhimurium* minicells and the cultured-cell plasma
121 membrane was ~50 nm, which matches the needle length of the injectisome imaged prior to their
122 application to cultured cells (Extended Data Fig. 2a-f, m). The orientation of the injectisomes in the
123 bacteria/target cell interface was perpendicular relative to the host PM, and the needle of the host-
124 interacting injectisomes appeared straight (Fig. 2c). We also observed that the interaction of the
125 injectisome and the target cell resulted in a noticeable inward bend of the PM (Extended Data Video
126 1). Consistent with this observation, the distance between the bacterial cell and the PM was shorter
127 (~30 nm) than the distance observed in areas immediately adjacent to the injectisomes (Extended
128 Data Fig. 2g-m). However, we did not observe any sign of penetration of the needle filament through
129 the host cell plasma membrane as it has been previously proposed (Hoiczyk & Blobel, 2001). The
130 length of the bacterial-envelope-embedded injectisome base substructure before (30.5 ± 2.3 nm) and
131 after (30.8 ± 2.2 nm) the bacteria/target cell interactions remained unchanged (Extended Data Fig.
132 2m). This is in contrast to the *Chlamydia* T3SS, which has been reported to undergo significant
133 conformational changes upon contact with host cells (Nans et al, 2015). The reasons for these
134 differences is unclear and may either reflect intrinsic differences between these T3SS, or differences
135 in the methodology used, which resulted in higher resolution of the visualized *S. Typhimurium* T3SS
136 structures. Together, these observations indicate that (1) the interactions of the T3SS injectisome with

137 the target cell results in the bending of the PM without penetration of the needle filament, and (2) upon
138 contact with target cells the injectisome does not undergo conformational changes that could be seen
139 at this level of resolution.

140

141 **Visualization of the formation of the translocon in the target host cell membrane**

142 The deployment of the translocon is an essential step in the T3SS-mediated delivery of
143 effector proteins. However, very little information is available on both, the architecture of the
144 assembled translocon, as well as the mechanisms leading to its deployment on the target cell. It is
145 believed that the deployment process must be initiated by a sensing step most likely mediated by the
146 tip complex (*i. e.* SipD), a step that must be followed by the subsequent secretion of the translocon
147 components (*i. e.* SipB and SipC) destined to be inserted on the target eukaryotic cell PM. To capture
148 the formation of the translocon, we analyzed over 600 injectisomes adjacent to the host PM.

149 Classification of sub-tomograms depicting the region of the tip complex (Fig. 3a) showed the PM at
150 various conformations and distances to the needle tip (Fig. 3b-i), which presumably represent
151 intermediate steps prior to the deployment of the translocon and the resulting intimate attachment of
152 the bacteria to the PM. After further alignment and classification of the injectisomes in intimate
153 association with the PM, we obtained a distinct structure of the putative translocon in the host PM
154 (Fig. 3j). Sub-tomogram averages of injectisomes from the *S. Typhimurium* translocase-deficient
155 mutants Δ sipB or Δ sipD in close proximity to the target cell PM did not show this distinct structure,
156 thus confirming that this density corresponds to the assembled translocon (Fig. 3k, l). To better
157 visualize the translocon in 3D, we segmented the distinct translocon structure in the context of the
158 host PM, the needle, and its tip complex (Fig. 3m, n). We found that the translocon has a thickness of
159 8 nm spanning the host PM and a diameter of 13.5 nm on its protruding portion (Fig. 3j). This size is
160 substantially smaller than reported size of the translocon of enteropathogenic *E. coli* assembled from
161 purified proteins *in vitro*, which was estimated to be 55-65 nm in diameter (Ide et al, 2001). One half
162 of the translocon is embedded in the host PM, while the other half protrudes towards the host

163 cytoplasm. In the middle of the protruded portion, we observed a hemispherical hole, which may
164 represent the channel through which effectors make their way into the target cell plasma membrane.
165 The presence of this structure is entirely consistent with the long-standing notion that the translocon
166 forms a conduit through the host PM to facilitate the translocation of effectors (Mueller et al, 2008).

167 Comparison of the arrangement of the injectisomes in relation to the target cell PM in wild-type
168 and translocase-deficient strains revealed marked differences. In comparison to wild-type, bacterial
169 cells obtained from translocase-deficient mutants showed a smaller proportion of injectisomes
170 attached to the host PM (Fig. 4a). We also noticed that, unlike wild-type injectisomes, which most
171 often appeared perpendicular to the target cell PM (Fig. 2c), the injectisomes from the translocase
172 deficient mutant strains $\Delta sipB$, $\Delta sipD$, or $\Delta sipBCD$ appeared arranged at various angles relative to the
173 PM. These observations are consistent with the fact that in the absence of the translocases, the
174 injectisomes do not intimately attach to the target cell PM (Fig. 4b-j, Extended Data Fig. 3). These
175 data also further support the notion that the distinct structure embedded in host membrane in close
176 apposition to the T3SS injectisome needle tip is formed by the translocon.

177 One of the striking features associated with the intimate T3SS mediated contact and the
178 formation of the translocon is the target cell PM remodeling around the translocon-injectisome needle
179 tip interface, appearing in a “tent-like” conformation (Fig. 2c, Extended Data Video 1). This feature is
180 likely the result of the close association between the bacteria and the target cell presumably mediated
181 not only by the T3SS but also by multiple additional adhesins encoded by *S. Typhimurium*. In fact,
182 the distance of the bacteria OM and the target cell is shorter than the length of the needle itself, which
183 results in the bending of the target cell PM and the “tent-like” conformation around the injectisome
184 target cell PM interface. It is possible that this intimate association may facilitate the T3SS-mediated
185 translocation of effector proteins (Fig. 5, Extended Data Video 2).

186

187 **DISCUSSION**

188

189 We have presented here a high-resolution view of the interface between the *S. Typhimurium*
190 T3SS injectisome and the target eukaryotic cell plasma membrane, which has provided details on the
191 intimate attachment of this pathogen that precedes T3SS-mediated effector protein translocation.
192 Notably, we observed a notable “bend” on the target cell PM in areas of the bacteria/PM interface
193 surrounding the needle filament. These observations reflect the intimate attachment that is known to
194 be required for optimal T3SS-mediated effector translocation that may result in the close apposition of
195 the tip complex and the target cell PM. Importantly, we have been able to visualize a distinct density
196 within the region of the target cell PM in close apposition to the needle tip of the T3SS injectisome.
197 We present evidence that this density corresponds to the deployed T3SS translocon since this density
198 was absent in the bacteria/PM interface of mutant bacteria that lack the translocon components. The
199 dimensions of this structure (~13.5 nm in diameter, 8 nm in thickness) are much smaller than previous
200 estimates (50-65 nm in diameter) obtained from the observation of EPEC translocons assembled from
201 purified components on red blood cells (Ide et al, 2001). It is unlikely that these differences may reflect
202 substantial differences between the dimensions of translocons from different T3SSs. It is possible
203 that the observed differences may reflect differences in the experimental approaches used in the
204 different studies. However, most likely these observations indicate fundamental differences in the
205 translocon assembly mechanisms from purified components in comparison to translocon assembly
206 during bacteria/target cell PM interactions. It is well established that the deployment of the translocon
207 during bacterial infections is orchestrated by the needle filament tip complex of the T3SS injectisome.
208 In the absence of the tip protein, the components of the translocon are very efficiently secreted but
209 they are unable to form the translocon (Kaniga et al, 1995; Ménard et al, 1994). It is therefore possible
210 that the insertion in the membrane of the purified translocon components in the absence of the tip
211 protein may lead to a structure that is substantially different from the one that results from the
212 interaction of bacteria with target cells.

213 Contrary to what has been previously proposed for the Chlamydia T3SS (Nans et al, 2015),
214 we did not observe any obvious conformational changes in the injectisomes prior and post interaction

215 with host cells. It is unlikely that these observations are an indication of fundamental differences
216 between the T3SS injectisomes in different bacteria. Rather, the differences observed might reflect
217 differences in the experimental approaches used in our studies, which resulted in a substantially
218 higher resolution.

219 In summary, our studies have provided a close-up view of the interface between the T3SS
220 injectisome and the target cell PM, which has resulted in the visualization of the deployed T3SS
221 translocon complex. Importantly, given the highly conserved nature of the T3SSs among many Gram-
222 negative bacteria, our studies have broad scientific implications and provide a paradigm for the study
223 host-pathogen interactions in a greater detail.

224

225 MATERIALS AND METHODS

226

227 **Bacterial strains.** The minicell producing *S. Typhimurium* $\Delta minD$, which is referred to in this
228 study as wild-type, has been previously described (Carleton et al, 2013; Hu et al, 2017). Mutations in
229 the genes encoding the translocases ($\Delta sipB$, $\Delta sipC$) or tip complex ($\Delta sipD$) proteins were introduced
230 into the $\Delta minC$ *S. Typhimurium* strain by allelic exchange as previously described (Lara-Tejero et
231 al, 2011).

232 **Isolation of minicells.** Minicell producing bacterial strains were grown overnight at 37 °C in
233 LB containing 0.3M NaCl. Fresh cultures were prepared from a 1:100 dilution of the overnight culture
234 and then grown at 37 °C to late log phase in the presence of ampicillin (200 µg/mL) and L-arabinose
235 (0.1%) to induce the expression of regulatory protein HilA and thus increase the number of
236 injectisomes partitioning to the minicells (Carleton et al, 2013). To enrich for minicells, the culture was
237 centrifuged at 1,000 x g for 5 min to remove bacterial cells, and the supernatant fraction was further
238 centrifuged at 20,000 x g for 20 min to collect the minicells. The minicell pellet was resuspended in
239 Dulbecco's Modified Eagles Medium (DMEM) prior to their application to cultured HeLa cells.

240 **HeLa cell culture on EM grid and infection.** HeLa cells were cultured in DMEM
241 supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). The day before plating, gold
242 EM grids with 2/1 Quantifoil were placed in glass bottom MatTek dishes (facilitating fluorescence
243 imaging and removal for cryo-preservation) and coated with 0.1 mg/ml poly-D-lysine overnight at
244 37°C. After rinsing the grids with sterile water, freshly trypsinized HeLa cells were plated on top of the
245 pre-treated grids that were allowed to grow overnight at 37°C/5% CO². To infect HeLa cells with *S.*
246 *Typhimurium* minicells, grids with adherent HeLa cells were removed from the culture dish and
247 minicells were directly applied to the grids.

248 **Vitrification and cryoEM sample preparation.** At different time points after infection, the EM
249 grids with HeLa cells and *S. Typhimurium* minicells were blotted with filter paper and vitrified in liquid
250 ethane using a gravity-driven plunger apparatus as described (Hu et al, 2017; Hu et al, 2015).

251 **Cryo-ET data collection and reconstruction.** The frozen-hydrated specimens were imaged
252 with 300kV electron microscopes. 713 tomograms were acquired from single-axis tilt series at ~6 µm
253 defocus with cumulative doses of ~80 e⁻/Å² using Polara equipped with a field emission gun and a
254 direct detection device (Gatan K2 Summit). 313 tomograms were acquired from single-axis tilt series
255 at ~1 µm defocus with cumulative doses of ~50 e⁻/Å² using Titan Krios equipped with a field emission
256 gun, an energy filter, Volta phase plate, and a direct detection device (Gatan K2 Summit). The
257 tomographic package SerialEM (Mastronarde, 2005) was utilized to collect 35 image stacks at a
258 range of tilt angles between -51° and +51° for each data set. Each stack contained 10-15 images,
259 which were first aligned using Motioncorr (Li et al, 2013) and were then assembled into the drift-
260 corrected stacks by TOMOAUTO (Hu et al, 2015). The drift-corrected stacks were aligned and
261 reconstructed by using marker-free alignment (Winkler & Taylor, 2006) or IMOD marker-dependent
262 alignment (Kremer et al, 1996). In total, 1026 tomograms (3,600 × 3,600 × 400 pixels) were generated
263 for detailed examination of the *Salmonella*-host interactions (Extended Data Table 3).

264 **Sub-tomogram analysis.** Sub-tomogram analysis was accomplished as described previously

265 (Hu et al, 2015) to analyze over 700 injectisomes extracted from 458 tomograms. Briefly, we first
266 identified the injectisomes visually on each minicell. Two coordinates along the needle were used to
267 estimate the initial orientation of each particle assembly. For initial analysis, $4 \times 4 \times 4$ binned sub-
268 tomograms ($128 \times 128 \times 128$ voxels) of the intact injectisome were used for alignment and averaging
269 by using the tomographic package I3 (Winkler & Taylor, 2006; Winkler et al, 2009). Then multivariate
270 statistical analysis and hierarchical ascendant classification were used to analyze the needle tip
271 complex (Winkler et al, 2009).

272 **3-D visualization and molecular modeling.** Outer membrane (OM) & inner membrane (IM)
273 of *S. Typhimurium*, Plasma membrane (PM) of HeLa cells, actin filaments, and ribosomes were
274 segmented using EMAN2 (Chen et al, 2017). UCSF Chimera (Pettersen et al, 2004) and UCSF
275 ChimeraX (Goddard et al, 2018) were used to visualize the sub-tomogram average structures in 3-D
276 and build atomic model of the T3SS injectisome. The atomic model was built as described briefly (Hu
277 et al, 2017) except for the basal body, which we docked PDB-5TCR (Worrall et al, 2016) and PDB-
278 3J1W (Bergeron et al, 2013). Video clips for the supplemental videos were generated using UCSF
279 Chimera, UCSF Chimera X, and IMOD, and edited with iMovie.

280 **Distance measurement and statistical analysis.** IMOD (3dmod Graph) was used to
281 measure lengths (in pixels) of various features. Each measurement was recorded in MS Excel for
282 statistical analysis: Mean, standard deviation, standard error of mean, and Welch's t-test.

283

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285

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288

289 **REFERENCES:**

290 Barta M, Guragain M, Adam P, Dickenson N, Patil M, Geisbrecht B, Picking W, Picking W (2012)
291 Identification of the bile salt binding site on IpaD from *Shigella flexneri* and the influence of ligand
292 binding on IpaD structure. *Proteins* **80**: 935-945

293

294 Bergeron JR, Worrall LJ, Sgourakis NG, DiMaio F, Pfuetzner RA, Felise HB, Vuckovic M, Yu AC,
295 Miller SI, Baker D, Strynadka NC (2013) A refined model of the prototypical *Salmonella* SPI-1 T3SS
296 basal body reveals the molecular basis for its assembly. *PLoS Pathog* **9**: e1003307

297

298 Blocker A, Deane J, Veenendaal A, Roversi P, Hodkinson J, Johnson S, Lea SM (2008) What's the
299 point of the type III secretion needle? *Proc Natl Acad Sci USA* **105**: 6507-6513

300

301 Carleton HA, Lara-Tejero M, Liu X, Galán JE (2013) Engineering the type III secretion system in non-
302 replicating bacterial minicells for antigen delivery. *Nat Commun* **4**: 1590

303

304 Chen M, Dai W, Sun SY, Jonasch D, He CY, Schmid MF, Chiu W, Ludtke SJ (2017) Convolutional
305 neural networks for automated annotation of cellular cryo-electron tomograms. *Nature methods* **14**:
306 983-985

307

308 Cheung M, Shen DK, Makino F, Kato T, Roehrich AD, Martinez-Argudo I, Walker ML, Murillo I, Liu X,
309 Pain M, Brown J, Frazer G, Mantell J, Mina P, Todd T, Sessions RB, Namba K, Blocker AJ (2015)
310 Three-dimensional electron microscopy reconstruction and cysteine-mediated crosslinking provide a
311 model of the type III secretion system needle tip complex. *Molecular microbiology* **95**: 31-50

312

313 Collazo C, Galán JE (1997) The invasion-associated type III system of *Salmonella typhimurium*
314 directs the translocation of Sip proteins into the host cell. *Mol Microbiol* **24**: 747-756

315

316 Deane JE, Roversi P, Cordes F (2006) Molecular model of a type III secretion system needle:
317 Implications for host-cell sensing. *Proc Natl Acad Sci USA* **103**: 12529-12533

318

319 Deng W, Marshall NC, Rowland JL, McCoy JM, Worrall LJ, Santos AS, Strynadka NCJ, Finlay BB
320 (2017) Assembly, structure, function and regulation of type III secretion systems. *Nature reviews
321 Microbiology* **15**: 323-337

322

323 Dietsche T, Tesfazgi Mebrhatu M, Brunner M, Abrusci P, Yan J, Franz-Wachtel M, Schärfe C, Zilkenat
324 S, Grin I, Galán J, Kohlbacher O, Lea S, Macek B, Marlovits T, Robinson C, Wagner S (2016)
325 Structural and Functional Characterization of the Bacterial Type III Secretion Export Apparatus. *PLoS
326 Pathog* **12**: e1006071

327

328 Galán JE, Lara-Tejero M, Marlovits TC, Wagner S (2014) Bacterial type III secretion systems:
329 specialized nanomachines for protein delivery into target cells. *Annual review of microbiology* **68**: 415-
330 438

331

332 Goddard TD, Huang CC, Meng EC, Pettersen EF, Couch GS, Morris JH, Ferrin TE (2018) UCSF
333 ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci* **27**: 14-25

334

335 Grosdent N, Maridonneau-Parini I, Sory M, Cornelis G (2002) Role of Yops and adhesins in
336 resistance of *Yersinia enterocolitica* to phagocytosis. *Infect Immun* **70**: 4165-4176.

337

338 Hoiczyk E, Blobel G (2001) Polymerization of a single protein of the pathogen *Yersinia enterocolitica*
339 into needles punctures eukaryotic cells. *Proceedings of the National Academy of Sciences of the*
340 *United States of America* **98**: 4669-4674

341

342 Hu B, Lara-Tejero M, Kong Q, Galán JE, Liu J (2017) In Situ Molecular Architecture of the *Salmonella*
343 Type III Secretion Machine. *Cell* **168**: 1065-1074 e1010

344

345 Hu B, Morado DR, Margolin W, Rohde JR, Arizmendi O, Picking WL, Picking WD, Liu J (2015)
346 Visualization of the type III secretion sorting platform of *Shigella flexneri*. *Proceedings of the National*
347 *Academy of Sciences of the United States of America* **112**: 1047-1052

348

349 Ide T, Laarmann S, Greune L, Schillers H, Oberleithner H, Schmidt M (2001) Characterization of
350 translocation pores inserted into plasma membranes by type III-secreted Esp proteins of
351 enteropathogenic *Escherichia coli*. *Cell Microbiol* **3**: 669-679

352

353 Kaniga K, Trollinger D, Galán JE (1995) Identification of two targets of the type III protein secretion
354 system encoded by the inv and spa loci of *Salmonella typhimurium* that have homology to the *Shigella*
355 IpaD and IpaA proteins. *Journal of bacteriology* **177**: 7078-7085

356

357 Kremer JR, Mastronarde DN, McIntosh JR (1996) Computer visualization of three-dimensional image
358 data using IMOD. *J Struct Biol* **116**: 71-76

359

360 Kubori T, Matsushima Y, Nakamura D, Uralil J, Lara-Tejero M, Sukhan A, Galán JE, Aizawa S-I
361 (1998) Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system.
362 *Science* **280**: 602-605

363

364 Lara-Tejero M, Galán JE (2009) *Salmonella enterica* serovar *typhimurium* pathogenicity island 1-
365 encoded type III secretion system translocases mediate intimate attachment to nonphagocytic cells.
366 *Infect Immun* **77**: 2635-2642

367

368 Lara-Tejero M, Kato J, Wagner S, Liu X, Galán JE (2011) A sorting platform determines the order of
369 protein secretion in bacterial type III systems. *Science* **331**: 1188-1191

370

371 Li X, Mooney P, Zheng S, Booth CR, Braunfeld MB, Gubbens S, Agard DA, Cheng Y (2013) Electron
372 counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM.
373 *Nature methods* **10**: 584-590

374

375 Loquet A, Sgourakis N, Gupta R, Giller K, Riedel D, Goosmann C, Griesinger C, Kolbe M, Baker D,
376 Becker S, Lange A (2012) Atomic model of the type III secretion system needle. *Nature* **486**: 276-279

377

378 Mastronarde DN (2005) Automated electron microscope tomography using robust prediction of
379 specimen movements. *J Struct Biol* **152**: 36-51

380

381 Ménard R, Sansonetti PJ, Parsot C (1994) The secretion of the *Shigella flexneri* Ipa invasins is
382 induced by epithelial cells and controlled by IpaB and IpaD. *EMBO J* **13**: 5293-5302

383

384 Misselwitz B, Kreibich SK, Rout S, Stecher B, Periaswamy B, Hardt WD (2011) *Salmonella enterica*
385 serovar *Typhimurium* binds to HeLa cells via Fim-mediated reversible adhesion and irreversible type
386 three secretion system 1-mediated docking. *Infect Immun* **79**: 330-341

387

388 Mueller C, Broz P, Cornelis G (2008) The type III secretion system tip complex and translocon. *Mol*
389 *Microbiol* **68**: 1085-1095
390
391 Nans A, Kudryashev M, Saibil H, Hayward R (2015) Structure of a bacterial type III secretion system
392 in contact with a host membrane in situ. *Nat Commun* ;**6**: 10114
393
394 Notti RQ, Stebbins CE (2016) The Structure and Function of Type III Secretion Systems. *Microbiol*
395 *Spectr* **4**
396
397 Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF
398 Chimera--a visualization system for exploratory research and analysis. *Journal of computational*
399 *chemistry* **25**: 1605-1612
400
401 Rathinavelan T, Lara-Tejero M, Lefebre M, Chatterjee S, McShan AC, Guo DC, Tang C, Galán JE, De
402 Guzman RN (2014) NMR model of PrgI-SipD interaction and its implications in the needle-tip
403 assembly of the *Salmonella* type III secretion syste. *J Mol Biol* **426**: 2958-2969
404
405 Schraadt O, Lefebre MD, Brunner MJ, Schmied WH, Schmidt A, Radics J, Mechtler K, Galán JE,
406 Marlovits TC (2010) Topology and organization of the *Salmonella typhimurium* type III secretion
407 needle complex components. *PLoS Pathog* **6**: e1000824
408
409 Schraadt O, Marlovits TC (2011) Three-dimensional model of *Salmonella*'s needle complex at
410 subnanometer resolution. *Science* **331**: 1192-1195
411
412 Winkler H, Taylor KA (2006) Accurate marker-free alignment with simultaneous geometry
413 determination and reconstruction of tilt series in electron tomography. *Ultramicroscopy* **106**: 240-254
414
415 Winkler H, Zhu P, Liu J, Ye F, Roux KH, Taylor KA (2009) Tomographic subvolume alignment and
416 subvolume classification applied to myosin V and SIV envelope spikes. *J Struct Biol* **165**: 64-77
417
418 Worrall LJ, Hong C, Vuckovic M, Deng W, Bergeron JR, Majewski DD, Huang RK, Spreter T, Finlay
419 BB, Yu Z, Strynadka NC (2016) Near-atomic-resolution cryo-EM analysis of the *Salmonella* T3S
420 injectisome basal body. *Nature* **540**: 597-601
421
422 Zierler MK, Galán JE (1995) Contact with cultured epithelial cells stimulates secretion of *Salmonella*
423 *typhimurium* invasion protein InvJ. *Infect Immun* **63**: 4024-4028
424
425

Figures and Figure Legends:

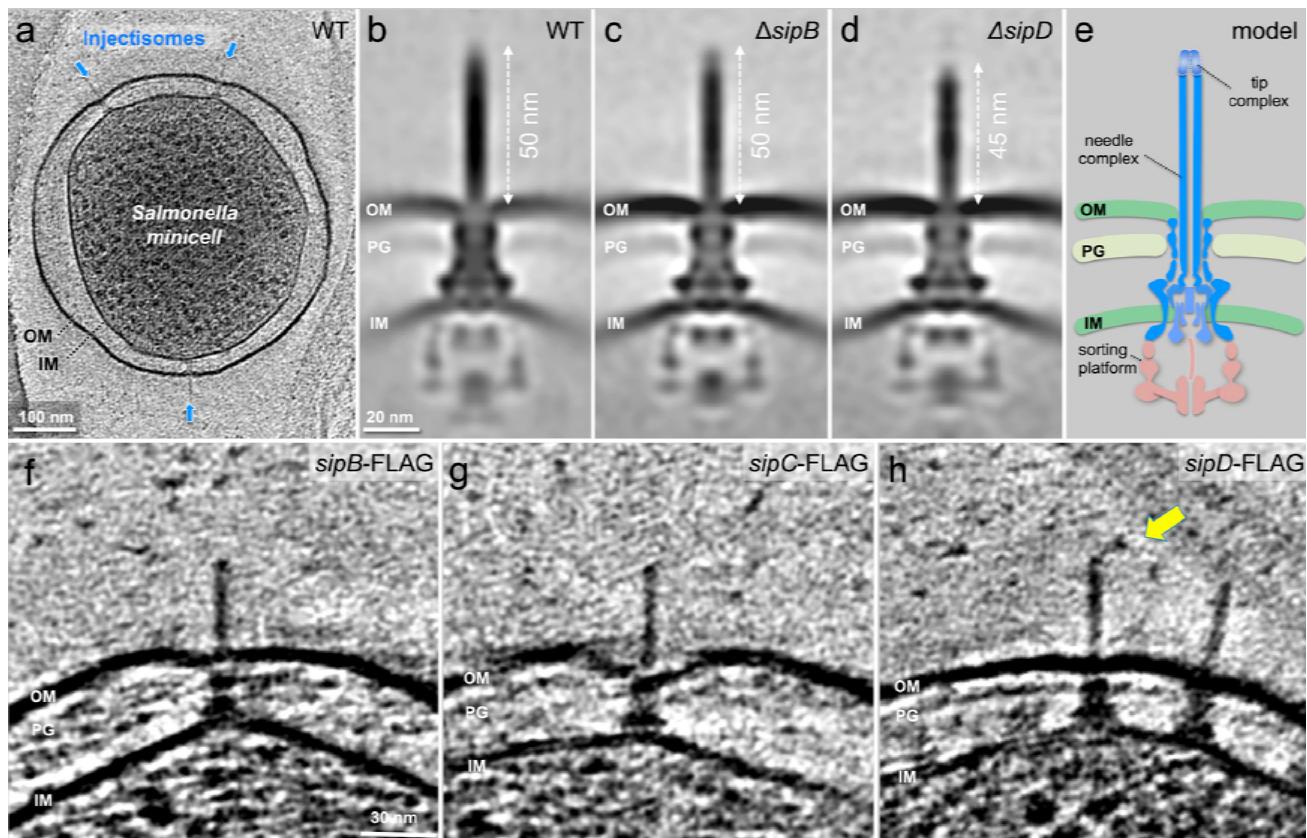


Figure 1. In situ structures of host-free *S. Typhimurium* T3SS injectisome in wild-type (WT), $\Delta sipB$, and $\Delta sipD$ minicells.

(a) A central section of a tomogram showing *S. Typhimurium* minicell containing multiple injectisomes.

(b-d) The central sections of sub-tomogram averages showing injectisomes of WT, $\Delta sipB$, and $\Delta sipD$, respectively. Outer membrane (OM), peptidoglycan (PG), and inner membrane (IM) of *S. Typhimurium* are annotated.

(e) A schematic of the injectisome.

(f-h) The central sections of tomograms showing injectisomes from strains expressing epitope-tagged (FLAG) SipB, SipC, and SipD, respectively. Yellow arrow indicates antibody bound to the epitope-tag.

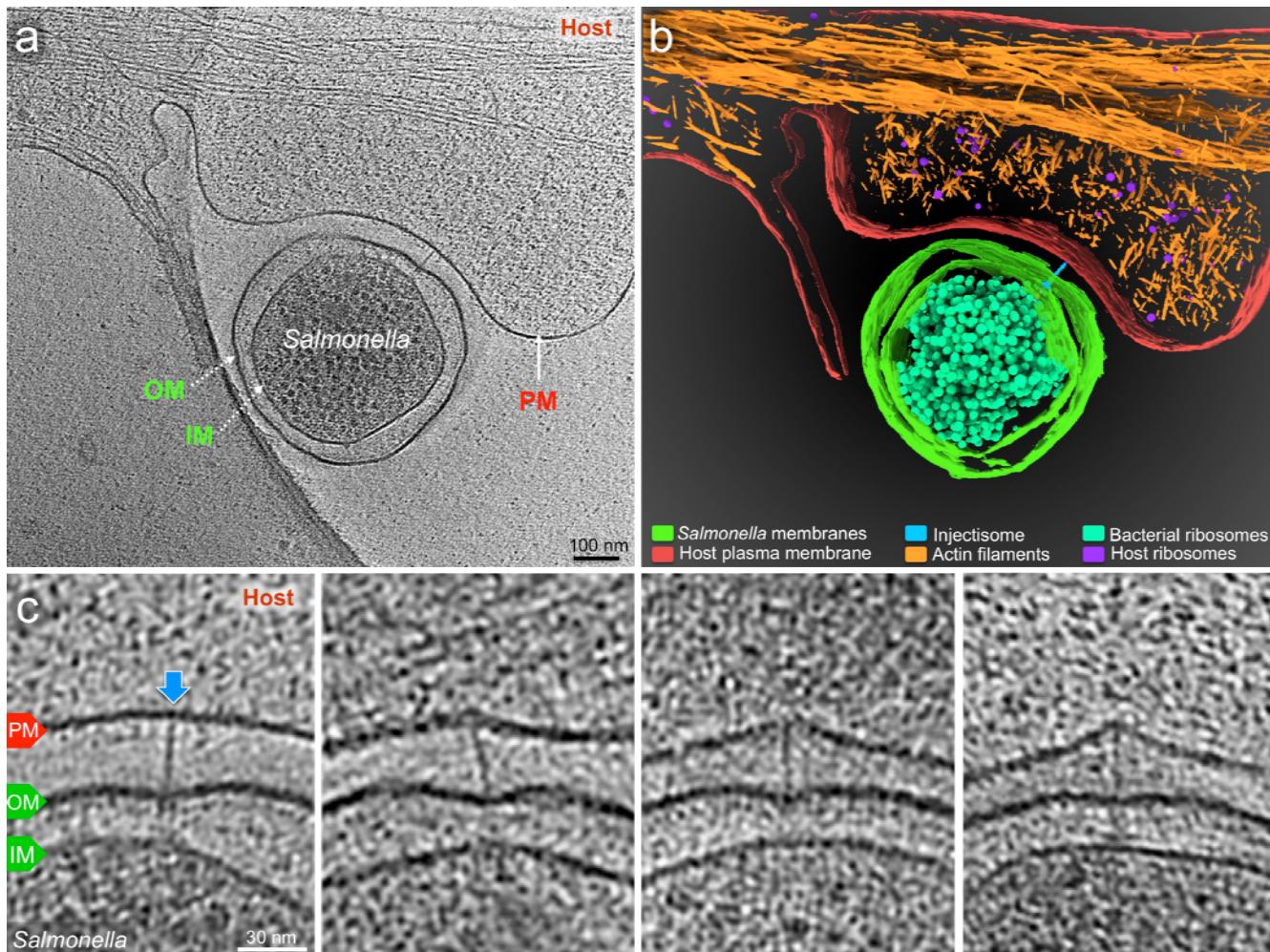


Figure 2. Visualization of the T3SS mediated *Salmonella*-Host interactions.

(a) A central slice showing a *S. Typhimurium* minicell interacting with a host. Plasma membrane (PM) of HeLa cell, outer membrane (OM) and inner membrane (IM) of *S. Typhimurium* are annotated.

(b) 3D rendering of the tomogram shown in **(a)**.

(c) Tomographic slices showing injectisomes interacting with the host PM. Blue arrows indicate needles attached to the host PM. Direction of the arrow represents the angle of needle perpendicular to the host PM.

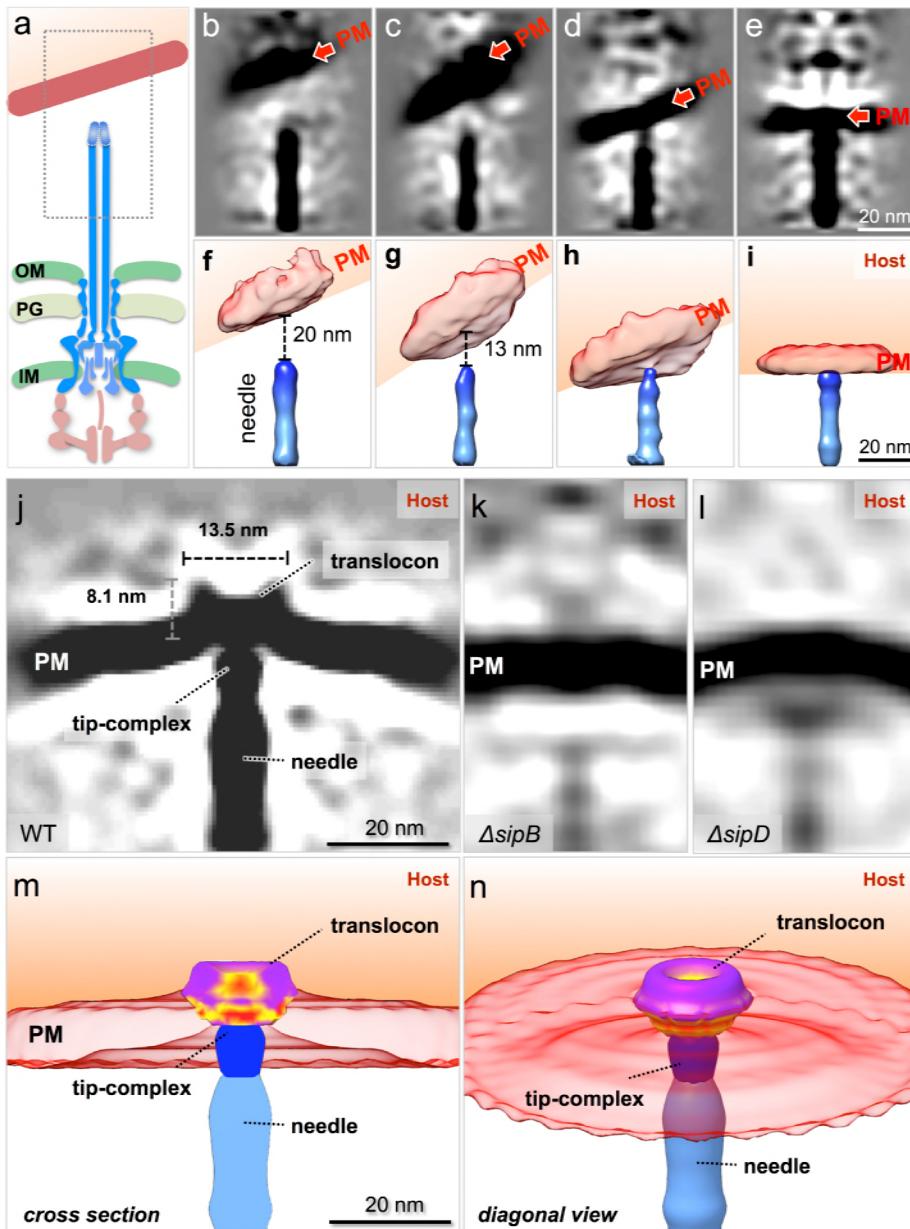


Figure 3. *In situ* structural analysis of the interface between the T3SS needle and the host membrane reveals a novel structure of the intact translocon.

(a) A schematic representation of the *S. Typhimurium* injectisome with a box highlighting the area used for alignment and classification

(b-e) Central sections and (f-i) 3-D surface views of class average structures showing different conformations of the needle - PM interaction.

(j-l) Central sections of the sub-tomogram average structures of the interface between the host PM and the needle of WT, $\Delta sipB$, and $\Delta sipD$, respectively. Surface rendering of the structure in panel j in (m) a cross-section view and (n) a diagonal view.

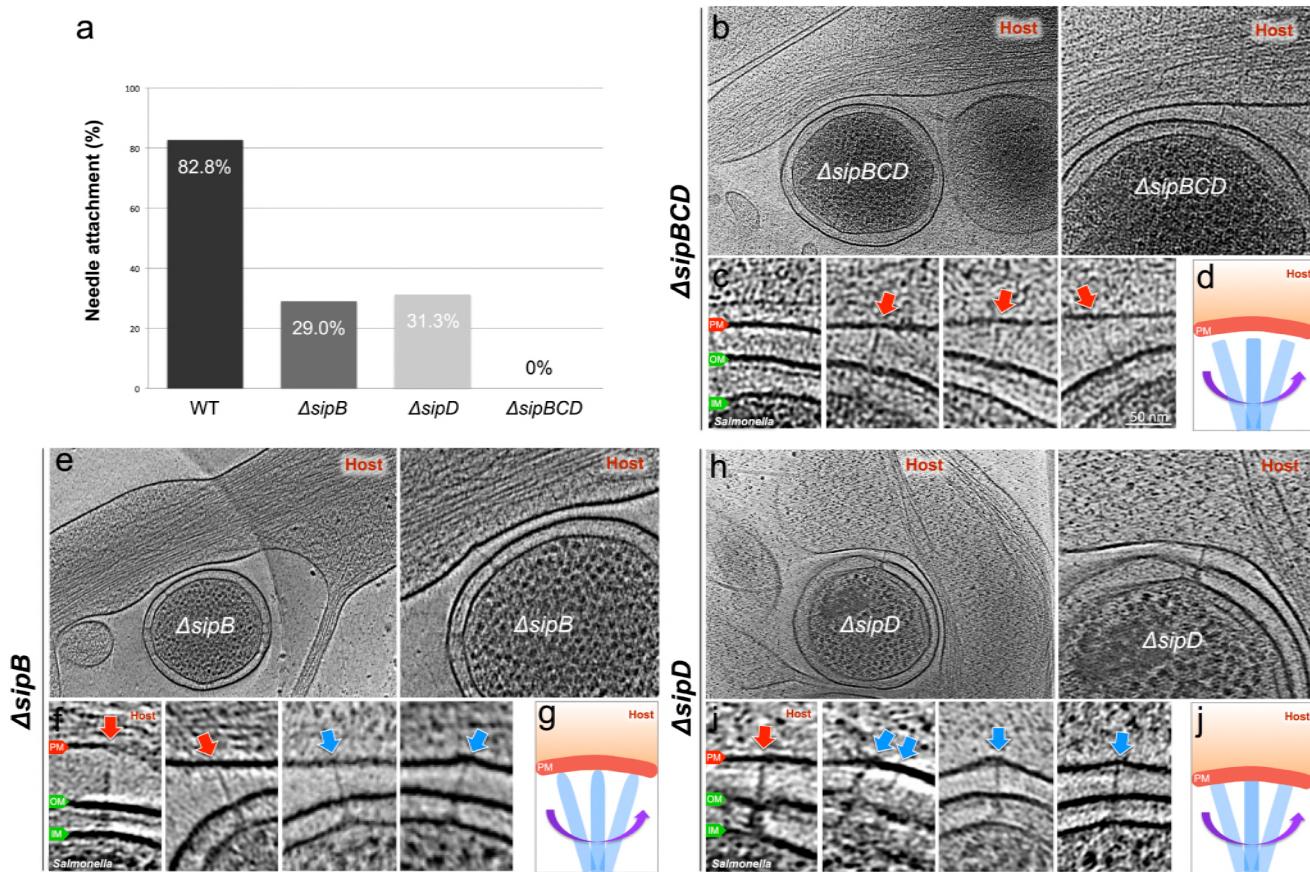


Figure 4. Deletion of the protein translocases disrupts the T3SS-dependent intimate attachment to the host PM, and the formation of the translocon.

(a) Percentage of minicells attached to the host membrane via needle-membrane contact

(b, c, e, f, h, i) Central slices from representative tomograms showing injectisomes interacting with the host PM. Blue arrows indicate needles attached to the host PM. Red arrows indicate unattached needles. Direction of the arrow represents the angle of needle perpendicular to the host PM.

(d, g, j) Schematic models depicting needle-attachment patterns.

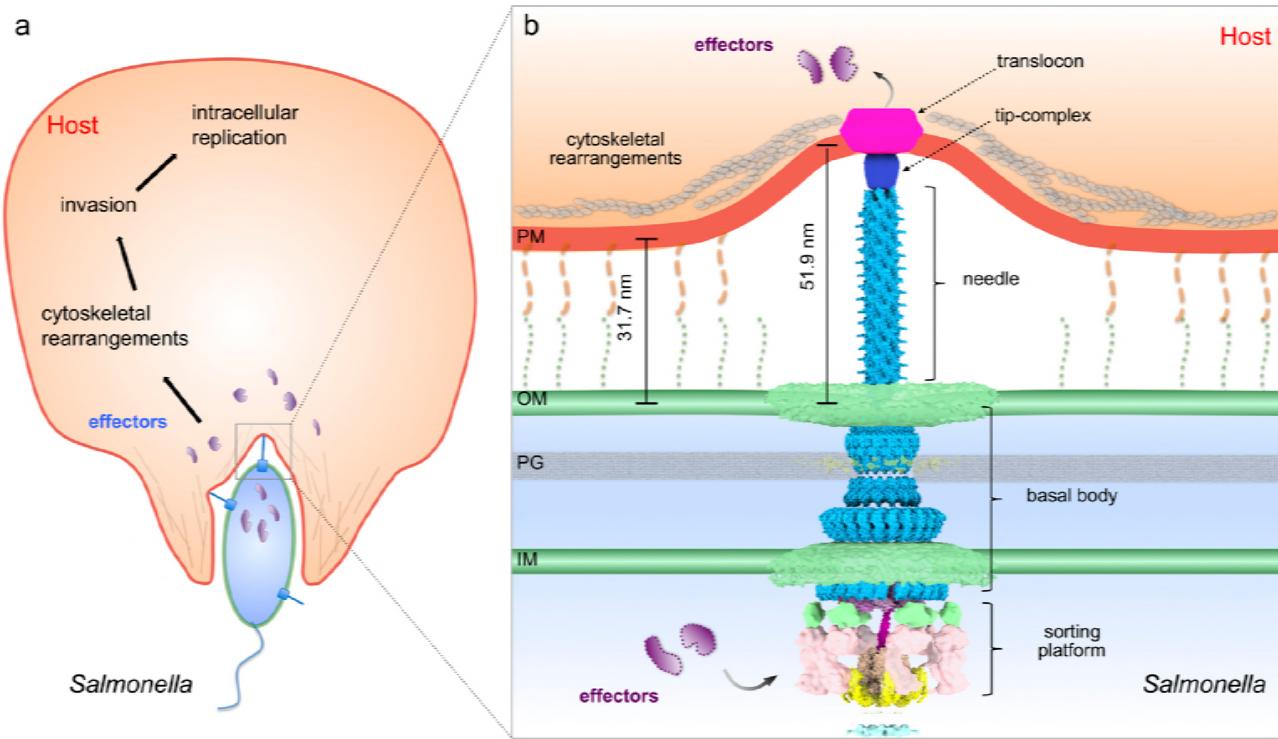


Figure 5. Model of the *S. Typhimurium* injectisome interacting with the host cell. (a) A schematic diagram of *S. Typhimurium* interacting with the host cell. (b) Molecular model of the T3SS injectisome at the *Salmonella*-host cell interface.