

Supplementary Figure 1. Fourier shell correlation curves for sub-tomogram averages and comparisons to other published *in situ* T3SS structures. **a**, Resolution estimates after applying Fourier shell correlation to independently processed half-data sets (gold-standard procedure, 0.5/0.143 criterion are both indicated. (upper graph) FSC curve for the host-free (red, 3.3 nm) and host-contact T3SS (blue, 3.8 nm) assuming 12-fold symmetry and using a mask to align the basal body and outer membrane. (middle graph) FSC curve for the host-free (red, 4.3 nm) and host-contact (blue, 4.6 nm) T3SS assuming 6-fold symmetry and using a mask to align the basal body, inner membrane, and sorting platform-ATPase in the cytosol. (lower graph) FSC curve for host-free *Salmonella* T3SS basal body assuming 12-fold symmetry. **b**, Side-by-side comparison of the T3SS from host-free *Chlamydia* to T3SS from the bacterial envelope of *Yersinia enterocolitica* (EMD-5694), *Shigella flexneri* (EMD-2667), and *Salmonella enterica* (Fig. 1d) mini-cells.

CdsC	531	GAIAAQAIQDIGYLYVTAMDEDFINTLNSIQWLEEVNNSIVVICNQANVDKVVSTLNGZ
Gspd_Vcholer	298	QPTTSKRN-----EVMIAAHADTNSLVLTAPQDIWAMLEVIGQL
Gspd_Koxyto	301	KPVAALDK-----NIIRKAGQTNALIVTAAPDVNDLERVIAQL
InvG	263	AAGN-----IKIVAYFDNSLLVKGTAEOVHFIFEMQVKAL
MxiD	260	ILED-----VSLIAYPETNSILVKCNDQQIQIIRDLITQL
YscC	247	-----VEADPSLNAIIVRDSPERIPMYQRILHAL
EscC	238	-----SVTISADPRLNAVVVVDREITLDIYQQITISEL
CdsC	591	DLPPKQVVEVLLETLSLEKSWDFGVQWAALGDEQGVAYASGLLSNTGLTDLPLRNQSLP
Gspd_Vcholer	338	DIRRAQVLREALIVEMAEGDGINLGVQWGSLESG--SVIQYNGTGASIGNVMIGLEEAKD
Gspd_Koxyto	341	DIRRQVLVEAIIAEVQDADGLNLGQWANKNAGM---TQFTNSGLPISTAIAGANQYNK
InvG	298	DVAKRHVELSLTWIVDLNKSLELERLGTSSWCSITIG-----TQFTNSGLPISTAIAGANQYNK
MxiD	295	DVAKRHVELSLTWIVDLNKSLELERLGTSSWCSITIG-----TQFTNSGLPISTAIAGANQYNK
YscC	276	DKPSARIEVALSIVDINADQLTELGVDNRVGIKTC-----TQFTNSGLPISTAIAGANQYNK
EscC	270	DIEQRQIEISVSIIDVDANDLQQLGVNNSCTLNAG-----TQFTNSGLPISTAIAGANQYNK
Secretin		
CdsC	651	VAPNPGNISLPTPGQLAGISDMMYGSSAFCLGIIGNVLSHNGKSYL-TLGGLLSALDQDG
Gspd_Vcholer	396	TTQTKAVYDTNNNFLRNETTTTKGDYTKLASATSSIQCAAVSIAMG-DWTALINAVNSDS
Gspd_Koxyto	398	DGTVS-----SSLASALSSFNCAAGFYQC-NWAMLTATSSST
InvG	333	-----DKLQVSLQSS-----ISTLDGSRFIAAVNALBEKK
MxiD	330	-----DSFGASFNMSSSASISTLDGKFPASVMALNOKK
YscC	311	-----NNHVQVVKTTGDQSNIASNGALGSLIDARGLDYLLARVNLLENEG
EscC	305	-----QGTIAFNSSTAQANISSSVISNASNFMIRVNALQONS
CdsC	710	DTTVLNFPRIMADTQOASFVFGQIIPFOITSTVIOETGSVTONTEYEDICVNLVVTSTH
Gspd_Vcholer	455	SSNLLSSPSTVMDNGEASFIVGEVPPVINGSTAGSNNDNPFQTVDRKEVGIKLRVVPQI
Gspd_Koxyto	436	KNDLIATPSIVTLNMEATFNVGQVPPVLAGSQTTSG-DNIFNVTVERKTVGIKLRVVKPQI
InvG	364	QAVVSRPVELLTOENVPATFDNNTFFYFLLIGERNVA-----LEHVTYGTMLRVLPFR
MxiD	364	KAVVSRPVELLTOENVPATFDNNTFFYFVSLVGERNS-----LEHVTYGTMLRVLPFR
YscC	356	SAQVSRPVELLTOENVAQVIDHSETFYVVTGKEVAE-----LKGITYGTMLRVLPFR
EscC	342	KAKILSOPSTITLNMQAALDKNVFFYTVSGEKVAS-----LESITSCTLRVTPRI
CdsC	770	APNNV-----VTLQIEQTISELHSAQ-----GVLTEVTDKTFAAARLQVFDGCF
Gspd_Vcholer	515	NEGNS-----VOLNIEQEVSNVLGAN-----GAVDVRFAKRQLNLSVMVQDQGM
Gspd_Koxyto	495	NEGDS-----VLEIEQEVSVADASS-----TSSDLGATFNTRTVNNAVLVGSGET
InvG	417	SADQ-----IEMSLDIED--GNDKTPQSDTTSVDALPEVGRLLISTIARVPHGKS
MxiD	417	SSRCQ-----IEMSLTIEDGTGNSQSNYNYNNTSVLPEVGRLLISTIARVPHGKS
YscC	409	LTOCDKS-----EISLNLHIEDGNQKPN-----SGIEGIPTSRTVVDVAVRGGHQS
EscC	395	LDDSSNSLTGKRRERVRLLDIQQDGNQSTNQSNQAQDASSTLPEVQNSEMTTEATLSAGES
CdsC	814	LVMSGHIRDKLTKIIVSGVPLLSLPLIKCLFSRSIDQROKRNIMIFIKPKVIVSSFEETA
Gspd_Vcholer	559	LVLGGLIDERALESSEKVPLLGDIPLLGLFRSTSSQVEKKNLWVFIKPTIIRDGVTDAG
Gspd_Koxyto	543	VVVGGLLDKSVSDTADKVPLLGDIPVIGALFRSTSKKVSRRNLMLFIRPPIVRDRDEYRQ
InvG	467	LLVGGYTRDANTDVSQIPFLGKPLIGSLFRYSKKNKSNVVRVEMIEPKRIVDPLTPDA
MxiD	469	LLVGGYTHETNSNEIISIPFLSSIPVIGNVFKYKTSNININIVRVELIQPREIKESSYNT
YscC	458	LIIGGIYRDELSVALSKVPLLDIPYLGALFRKSELTRRTRVRIIEPRILIDEGIAHLL
EscC	455	LLVGGFIQDKESSKDGIPLLSDIPVIGSLFSSVVKOKHSVVRVFLIKRVPFKKSASSE--
CdsC	874	LSNTEGYRYNWESERGS-----LEVAQRHAPCOHIPKVOLESNFKMLEIEA
Gspd_Vcholer	619	ITQRKYNIRAEQLFRA--EKGLRLDDASVPPVLEKFGDDRRHSPEIQFIE-QMEAKQ
Gspd_Koxyto	603	ASSGQYTAFNDAQSKQRGKENDDAMSNLDLEIYD--RQDAAFRQVSAID-AFNLGGN
InvG	527	SES-----VNNILKQSGAWSGDDKQKQVVRVVD--RGQEAIK-----
MxiD	529	AEY-----KSLISER--EIQKTTQIIPSETTLE--DEKSLVSYLNY-----
YscC	518	ALGNGQDLRTGILAVDEISNQSTTENKLLGGSQCPLNKAQEVQKWLSONKSSYLQCK
EscC		-----
CdsC	921	E-----
Gspd_Vcholer		-----
Gspd_Koxyto	660	L-----
InvG		-----
MxiD		-----
YscC	578	MDKSLGWRVVEGACTPAESWCVSAPKRGVL
EscC		-----

Supplementary Figure 2. Domain architecture of CdsC and YscC and the corresponding multiple sequence alignment of type II and type III secretin homologues (GspD and SctC). The *Chlamydia* secretin, CdsC, contains an N-terminal domain of 250 amino acids (dark red) in addition to the classical N-domains (yellow) found in other type II or type III secretins. CdsC

also uniquely contains type-II specific insertions throughout the N-domains, in the secretin domain (orange) and in the N3 loop (red). The predicted signal-peptide is also indicated (purple). Multiple-sequence alignments were produced by ClustalW Omega and graphically illustrated with BOXSHADE. Identical residues are shaded in black and similar residues in grey. Colored annotations in the sequence alignment correspond to the domains in the secretin schematic. Accession numbers are listed for each bacterial species. CdsC, *C. trachomatis* (M9UG25); GspD, *V. cholera* (C3LSG0); GspD, *K. oxytoca* (A0A068H9C0); InvG, *S. enterica* (P35672); MxiD, *S. flexneri* (Q04641); YscC, *Y. enterocolitica* (Q93KT1); EscC, enteropathogenic *E. coli* (B7UMB3).

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CdsP      1  -----MELNKTSESLFSAKIDHNHPRTEAHEPR-----
YscP      1  MNKITTTRSPLEPEYQPLGKPHHDLQARVDFEQALLHNKDNRRHPKEEPRRPVVRPHDLGKKEGQKGDGLRAH
EscP      1  -----
Spa32     1  --MALDNINLNFSSDKQIEKCEKLSIDNIDSLVLKRRKRVKVEIPEYSLIASN-----
SpaN/InvJ 1  ---MGDVSASVSSSGNILLPQQDEVGGLSEALKKAVEKHKTEYSGDKKDRDYGDAFVMHKETALPLLLLAAW

CdsP      29 -----DQREVRVFSLE--GRSSTRQEKADRMPGRRTSSRQESK-----
YscP      71  APLAATFQPGREVGKLPQHNNHONNHDfnLSPLAEGATNRKHLYQDSSRFDDRVESINALMPLAFFLEGV
EscP      1  -----MTRVSLKRNLIEPVSFRRPSD-----
Spa32     51  -----YFTIDKHFEHKKDKGEIYSGIKNAFELRNERATYSDIPES-----
SpaN/InvJ 68  R-----HGAPAKSEHNGNVSGLHHNGKSELRIAEKLLKVTAEKSVGLISAEAKVDKSAAL

CdsP      65  SSEEGAVHESSTAG-----VSSKEEESKGDGFFTGGNPTSG-----
YscP      141  TCETGTSSSESPCEFFGHDELQVQSPIDSAQPVQLNTRKPTVQSLNPAADGAEVMIWSVGRDRTLASIANKQ
EscP      21  -----DWPGQEESEFNDIFYHIKKRSPD-----
Spa32     91  MAIKENILIPDQD-----IKAREKINIGDMRGIFSYKSGN-----
SpaN/InvJ 124  LSSKNRPLESVSG-----KKLSADLKAVESVSEVTDNATGIS-----

CdsP      101 -----MALVETPMAVSEAMMETSMTVS-----QVDLQWVEQLVTSTVES-----
YscP      211  RDSRQKRLAEEPLPLHQEALPEVCPPAVSTTPDDHLVARWCATPVAEVAEK SARFPHKATVQSEQLDMTE
EscP      43  -----TIFIR-----MASLWKRLFYSSGRRR-----
Spa32     127 -----ADKNFERSHSSVNPDLNLES DNRNGQIGLKNHSL S-----
SpaN/InvJ 161 -----DDNIKALPGDNKAIAGEGVRKEGAPLAR DVAPARMAAANTGKPED-----

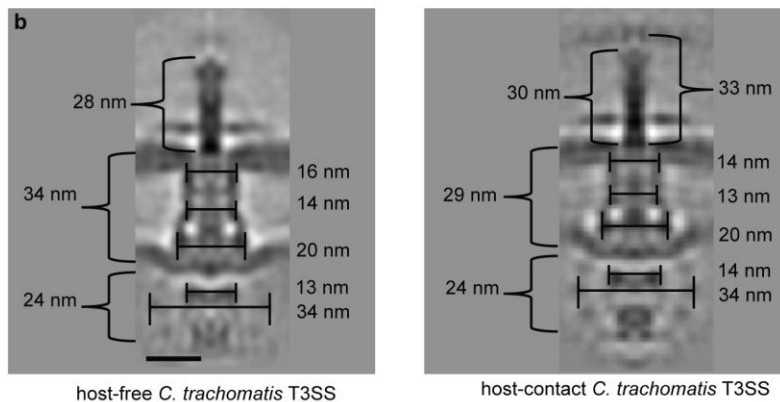
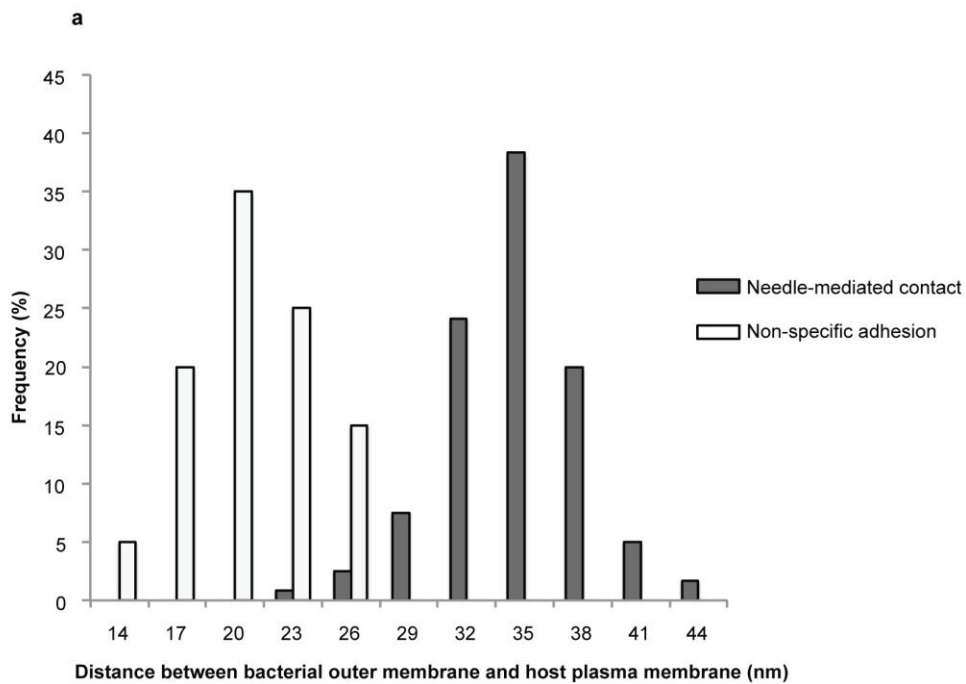
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YscP      281  LADRSQHLTDGVDSSKDTIEPPRPEELLPLREETLPPEMYSLSFTAPVITPCDHLLATMRATR-LASVSEQ
EscP      64  -----RYFEEGEHSFSILCGRLRGVVLTIKCSNGIYLSIK-ISO NNRN
Spa32     163 -----IDKNIADIISLNGSVAKSFEFPVMNKNTADITPMSMSLQ-EKSIVEN
SpaN/InvJ 206 -----KDHKVKVDVSQLPLQPTTIADTISQLTGCDEKMPLAAQSKPMMTIFPT

CdsP      197  ATQLVQONPKQLVSLVESLKAQNLNLTTELVV-GNAVAVSLPTIE-KIETPLHMIAATIRHHQ-----
YscP      350  LIQLAQLRLAVELELRGGSSQVTQLHLNLPPEL-GAIMVRIAEIPGKLHVELIASRETLRILAQGSYDLLE
EscP      107  HVFLYHKKDYVFDKLEIFPDEAIEFTIEYE-N-----
Spa32     209  DKNVFKKNSEMTHYHFKQWAGHSVSVISVSESG-SFVLPKPSDQFVGNKLDLILKQDAEG-----
SpaN/InvJ 253  ADGVKGEDSSLT YRFQRWGNDSVNIQARQAGEFSLIPSN TQVEHRLHDQWQNGNPQ-----

CdsP      257 -----EGDQEGEGRQDQHGGQHQEKKVFAHI
YscP      419  LQRIEPTQLDFQASGDSEESRQKRHVYEEWFAEE-
EscP      -----
Spa32     265 -----NYRFDSSQHNKGNKNNSTGYNEQSEEC-
SpaN/InvJ 310 -----RWHLTRDDQONPQQQQRHQQSGEEDDA

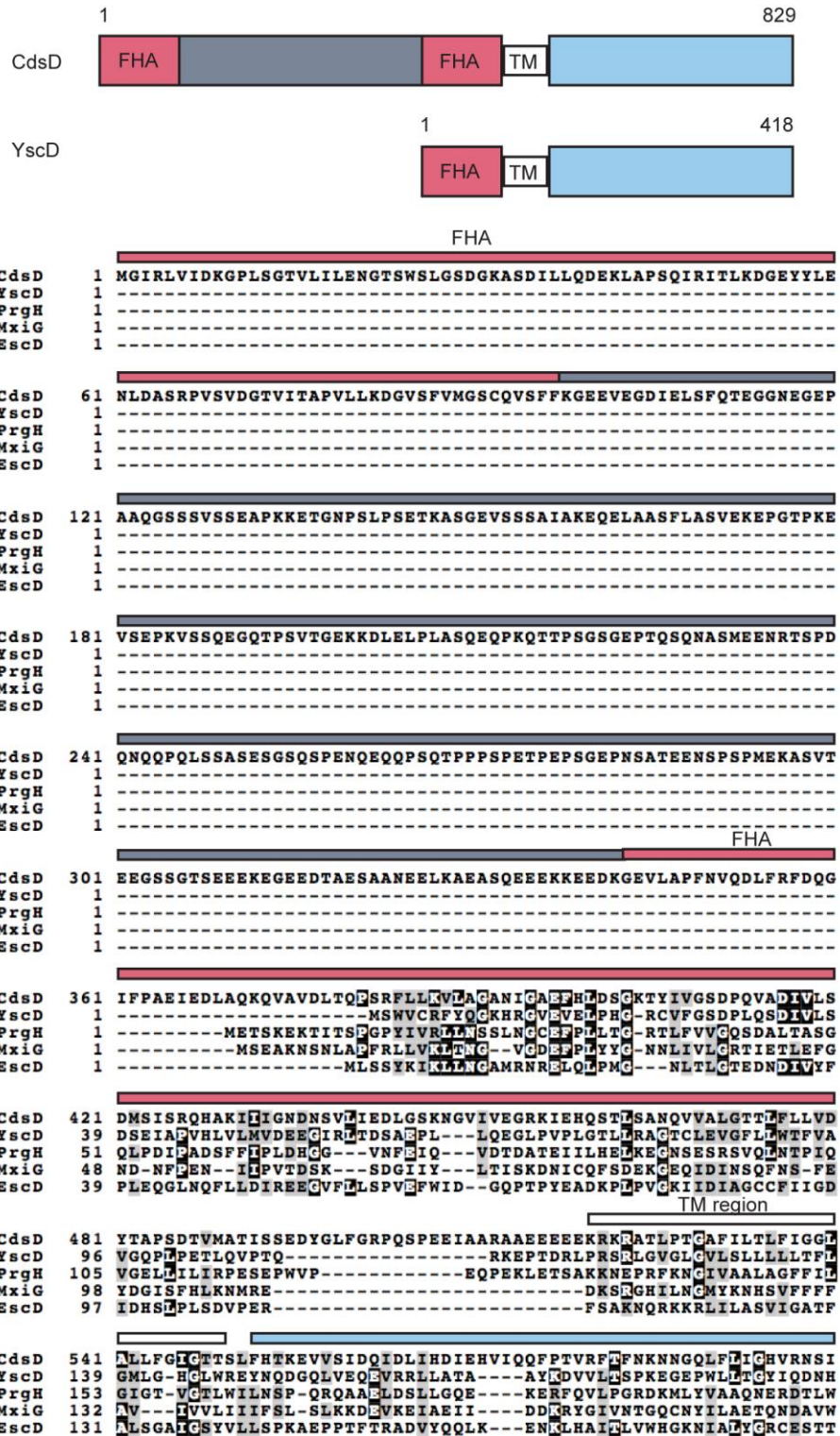
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Supplementary Figure 3. Multiple sequence alignment of tape measure protein SctP. The tape measure protein SctP regulates the needle length in T3SS-containing bacterial pathogens. Residue length and accession numbers are listed for each bacterial species. CdsP, *C. trachomatis* (283, M9UDI3); YscP, *Y. enterocolitica* (453, Q93KT6); EscP, enteropathogenic *E. coli* (138, B7UMA4); Spa32, *S. flexneri* (292, D2AJK8); SpaN/InvJ, *S. enterica* (336, P40613). Multiple sequence alignments were produced by ClustalW Omega and graphically illustrated with BOXSHADE. Identical and similar residues are shaded in black and grey respectively.



Supplementary Figure 4. Distance between bacterial outer membrane and target host

membrane. a, Histogram of length measurements for needle-mediated contact (average 33 ± 4 nm; range 22-44 nm; $n = 120$) and non-specific adhesion (average 20 ± 3 nm; range 14-25 nm; $n = 20$). Measurements in both cases were recorded manually from representative tomogram slices in IMOD **b**, Dimension comparisons for host-free and host-contact *Chlamydia* T3SSs.




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CdsD 601 DKSE[LYKVDALS FVKS VDDN VIDDEAVWQEMN ILLSKNPEFKGISMQSPEP]CIFVIS[CY
YscD 194 [RLS]QN[FLESHGIPFRLELRSMEE]LRQGAEFILQRLG[HG]EVS--LAPQAC[HW]QLN[CE
PrgH 207 [RQV]LARGDYD[NNARVINENEENKR]SIWLDTYYPQLA[LYR]HFD-EPRKPVFW[LSRQRN
MxiG 184 [SVA]NKTGFT[RCRYILVSNKEINR]QQYINQRFPFINLYVLNLVSDKAE[LLV]FSKERN
EscD 188 DLTPFFNYLKE[NNIFYYNKI ICNNO]ISAIN[DV]LTEY[GD]KD[II]IT--KGNKP[GF]LLS[CY

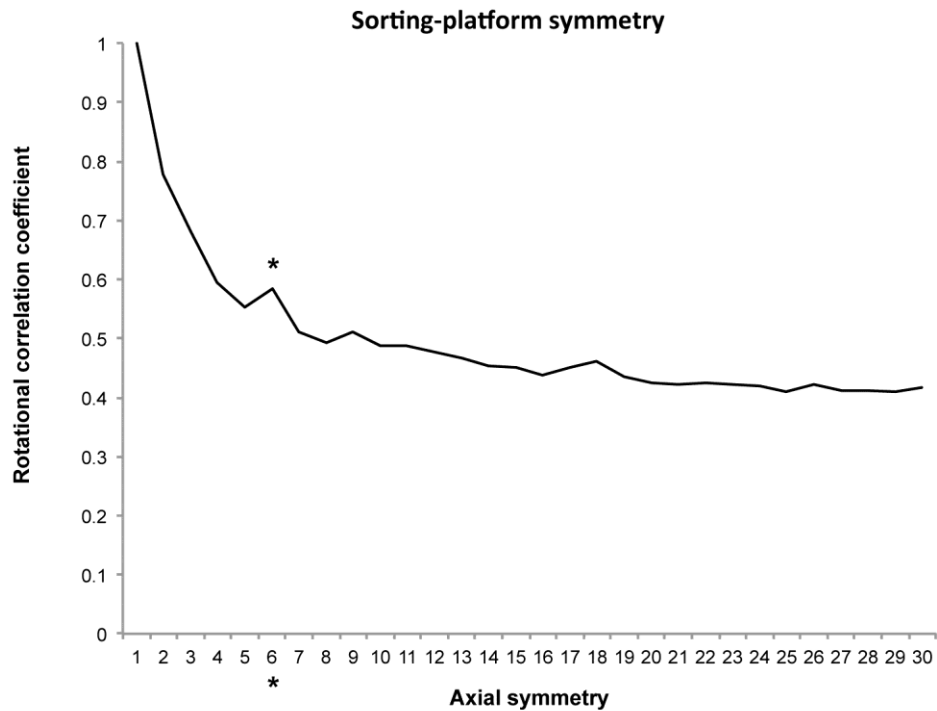
CdsD 661 LKTEEQAAC[ADY]N[LFHN]LSLLDNKV[IES]QVMKA[AGH]V[OS]GFANVHVSFTN[CEAV
YscD 252 VSEEIQKQK[IDS]LQAEV[GL]LGVENKVRIAG[QRKR]DAL[EG]G[TD]SDSFTVNVK[CELI
PrgH 266 TMSKKELEV[SK]LRALMP[ADSVNITLMD]DVTAAGQAEAG[KK]Q[AL]PYSRRNHKG[GVTF
MxiG 244 SSKDTELDR[KN]ALIVEFPY[IKN]IKFN[YS]DH[ARG]DAKGIFTKVNVQYKEICENKVTY
EscD 246 IPPSPKWSEVEN-[LL]NT[GV]AGWEIHNNSN-[NK]INE[ASE]FKKNI[IN]YVNI[FK]KNDVI

CdsD 721 LT-[CY]INNKDADKFR[TV]VQELQDIA[CI]RAVKNFVVLPAEEGVIDLNMRYPGRYRVTGFS
YscD 312 ELRQ[VND]EKLS[SN]QLQQTFRQEF[CN]RPKLELVN[VG]GQPQ-----HDELN[EV]QVAIS
PrgH 326 VIQ[AL]DDVEILRRARQFVDSYR[TV]GGR-----YVQFAE-----LKDDW[LC]RSFQ
MxiG 304 SVREELT[DE]KLELINRLISEHKNIY[CD]Q-----YIEFSVL-----LIDD[DF]CKSYL
EscD 304 IVAG[EV]SQQNESKILAIINAMNKNSNVK-----ILFQNI[QP]-----YISAD[IF]PKILR

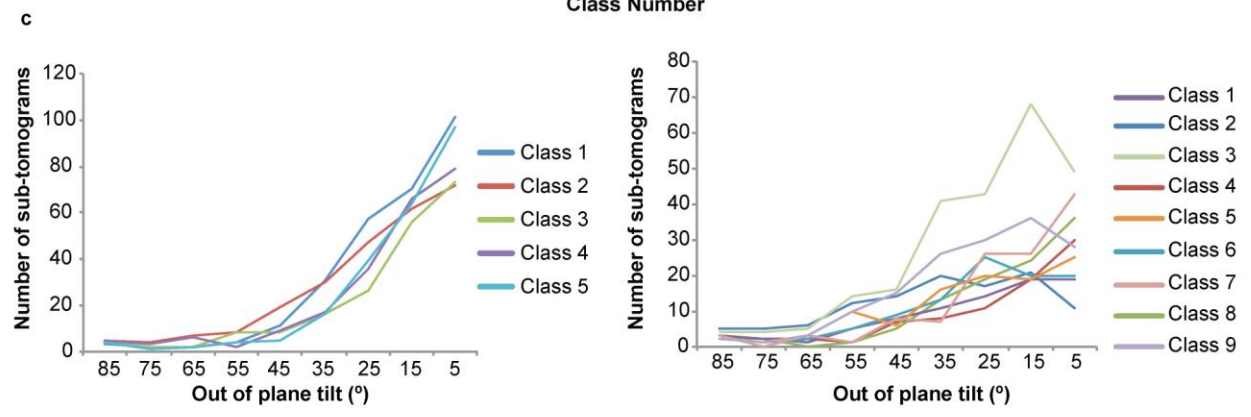
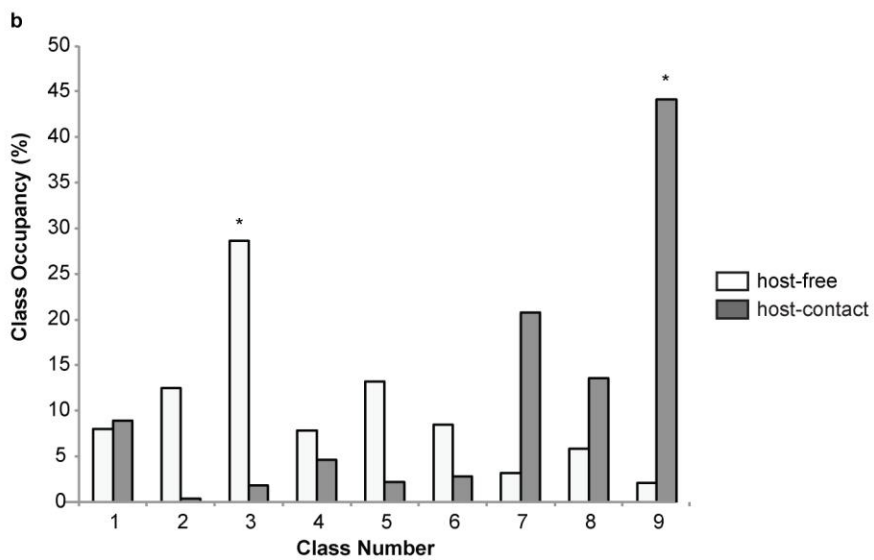
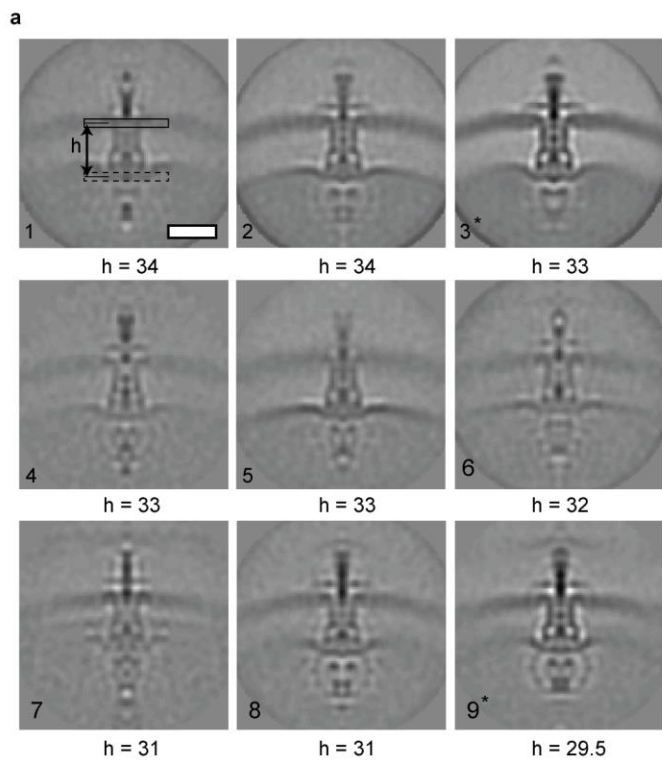
CdsD 780 KC---GDIS[IN]VVVNGRILTRGDILD[MT]VTS[QP]-HCIFLEREGLK[KK]EYNK---
YscD 365 LG---[KVP]V[V]DNHQRYPEGAILNN[CV]RLA[LR]-DAVIVSKGKREFV[QL]NGGKPR
PrgH 373 YG---AEG[IK]MSPGHWFPSPL-----
MxiG 351 NS---[KDS]V[M]NDKHWF[FD]KNN-----
EscD 353 ISGTM[KN]PT[AL]DNGTSLGIGSILK[CV]YVDA[DP]KDGINISRPDEYI[H]PLSY---

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Supplementary Figure 5. Domain architecture of major inner membrane proteins CdsD and YscD and multiple sequence alignment of SctD homologues. The *Chlamydia* inner membrane protein, CdsD, contains an additional N-terminal segment of 360 residues (pink and grey) including an extra cytoplasmic FHA domain. Colored annotations in the sequence alignment correspond to the domains in the CdsD/YscD schematic. Accession numbers are listed for each bacterial species. CdsD, *C. trachomatis* (M9UG17); YscD, *Y. enterocolitica* (Q01245); PrgH, *S. enterica* (P41783). MxiG, *S. flexneri* (P0A221); EscD, enteropathogenic *E. coli* (B7UM96). Multiple sequence alignments were produced by ClustalW Omega and graphically illustrated with BOXSHADE. Identical and similar residues are shaded in black and grey respectively.



Supplementary Figure 6. Symmetry analysis of *Chlamydia* T3SS sorting platform. To detect symmetry in the sorting platform, a masked asymmetric average was compared to a similarly masked average that had been rotated axially by intervals corresponding to rotational symmetries from c1 to c30. The cross-correlation coefficient was plotted as a function of the corresponding symmetry. Peaks along the cross-correlation coefficient plot will be observed when a rotated average exhibits a high degree of similarity with the unrotated average, demonstrating rotational symmetry.



Supplementary Figure 7. Multi-reference alignment classification of *Chlamydia* sub-

tomograms. Sub-tomograms were initially separated into 10 classes by multi-reference

alignment. One of the 10 class averages contained mostly noise and thus has been eliminated. **a,**

Central slices through 9 class averages of *Chlamydia* T3SSs. The class averages are sorted by

basal body height (h), which varies from 34 to 29.5 nm. **b,** Distribution of host-free and host-

contact *Chlamydia* T3SSs across the 9 classes depicted in (a). The most populated classes are

marked with an asterisk. Host-free T3SSs were mainly present in classes with elongated basal

bodies (classes 1-6). Host-contact T3SSs were mainly present in classes with short, compact

basal bodies (classes 7-9). **c,** Out of plane tilt of all sub-tomograms using MRA with 5 classes

(left, and as shown in Fig. 3) and with 9 classes (right, as shown in (a)). There is no predominant

bias in the distribution of particles into classes based on their out of plane tilt angle. Scale bar, 30

nm.