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***In Situ* Visualization of the pKM101-Encoded Type IV Secretion System Reveals a Highly Symmetric
ATPase Energy Center at the Channel Entrance**

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Short Title: *In situ* structure of the pKM101-encoded T4SS

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27 **Abstract (250)**

28 Bacterial conjugation systems are members of the type IV secretion system (T4SS) superfamily. T4SSs can be
 29 classified as ‘minimized’ or ‘expanded’ based on whether assembly requires only a core set of signature subunits
 30 or additional system-specific components. The prototypical ‘minimized’ systems mediating *Agrobacterium*
 31 *tumefaciens* T-DNA transfer and conjugative transfer of plasmids pKM101 and R388 are built from 12 subunits
 32 generically named VirB1-VirB11 and VirD4. In this study, we visualized the pKM101-encoded T4SS in the
 33 native context of the bacterial cell envelope by *in situ* cryoelectron tomography (CryoET). The T4SS_{pKM101} is
 34 composed of an outer membrane core complex (OMCC) connected by a thin stalk to an inner membrane complex
 35 (IMC). The OMCC exhibits 14-fold symmetry and resembles that of the T4SS_{R388}, a large substructure of which
 36 was previously purified and analyzed by negative-stain electron microscopy (nsEM). The IMC of the *in situ*
 37 T4SS_{pKM101} machine is highly symmetrical and exhibits 6-fold symmetry, dominated by a hexameric collar in the
 38 periplasm and a cytoplasmic complex composed of a hexamer of dimers of the VirB4-like TraB ATPase. The
 39 IMC closely resembles equivalent regions of three ‘expanded’ T4SSs previously visualized by *in situ* CryoET, but
 40 strikingly differs from the IMC of the purified T4SS_{R388} whose cytoplasmic complex instead presents as two side-
 41 by-side VirB4 hexamers. Together, our findings support a unified architectural model for all T4SSs assembled *in*
 42 *vivo* regardless of their classification as ‘minimized’ or ‘expanded’: the signature VirB4-like ATPases invariably
 43 are arranged as central hexamers of dimers at the entrances to the T4SS channels.

44

45 **Significance (113)**

46 Bacterial type IV secretion systems (T4SSs) play central roles in antibiotic resistance spread and virulence. By
 47 cryoelectron tomography (CryoET), we solved the structure of the plasmid pKM101-encoded T4SS in the native
 48 context of the bacterial cell envelope. The inner membrane complex (IMC) of the *in situ* T4SS differs remarkably
 49 from that of a closely-related T4SS analyzed *in vitro* by single particle electron microscopy. Our findings
 50 underscore the importance of comparative *in vitro* and *in vivo* analyses of the T4SS nanomachines, and firmly
 51 support a unified model in which the signature VirB4 ATPases of the T4SS superfamily function as a central
 52 hexamer of dimers to regulate substrate entry into and passage through T4SS channels.

53

54 Introduction

55 Many species of bacteria deploy type IV secretion systems (T4SSs) to deliver DNA or protein substrates to target
 56 cells (1-3). T4SSs designated as ‘minimized’ systems are assembled from a core set of signature subunits, while
 57 others termed ‘expanded’ are compositionally and structurally more complex, possibly reflecting adaptations
 58 arising over evolutionary time for specialized functions (3). In Gram-negative species, ‘minimized’ systems are
 59 assembled from ~12 subunits generically named VirB1 - VirB11 and VirD4 based on the paradigmatic
 60 *Agrobacterium tumefaciens* VirB/VirD4 T4SS (3). Three subunits (VirB7, VirB9, C-terminus of VirB10)
 61 assemble as an outer membrane core complex (OMCC) that spans the distal region of the periplasm and outer
 62 membrane (OM) (4). Four integral membrane components (VirB3, VirB6, VirB8, N-terminus of VirB10) and
 63 two or three ATPases (VirB4, VirD4, +/- VirB11) with catalytic domains located in the cytoplasm, together
 64 comprise the inner membrane complex (IMC) (5, 6). Some T4SSs elaborate an extracellular organelle termed the
 65 conjugative pilus from homologs of the VirB2 pilin and VirB5 pilus-tip subunit (3). ‘Expanded’ systems are
 66 composed of homologs or orthologs of most or all of the VirB/VirD4 subunits plus as many as 20 components
 67 that are system-specific (3).

68
 69 To better understand the mechanism of action of T4SSs and the structural bases underlying functional diversity,
 70 there is growing interest in solving the structures of intact machines or machine subassemblies. OMCCs are
 71 generally stable and amenable to purification, and structures are now available for OMCCs from several
 72 ‘minimized’ and two ‘expanded’ systems at resolutions approaching ~3 Å (4, 6-12). Structural analyses of inner
 73 membrane (IM) portions of T4SSs have been considerably more challenging likely due to dissociation or
 74 distortion of these regions of T4SSs during purification. Presently, one structure exists for a ‘minimized’ system
 75 encoded by the conjugative plasmid R388. Designated the VirB₃₋₁₀ complex, this structure was obtained by
 76 overproduction of the VirB3 - VirB10 homologs, affinity purification of the detergent-solubilized complex, and
 77 analysis by negative-stain electron microscopy (nsEM) (6). The VirB₃₋₁₀ complex consists of the OMCC and IMC
 78 connected by a thin, flexible stalk. The IMC is composed of a highly asymmetric IM platform connected to two
 79 side-by-side hexamers of the VirB4 ATPase extending into the cytoplasm. In an updated structure, two dimers of
 80 the VirD4 ATPase were shown to integrate between the VirB4 barrels (13).

81

82 IMCs of ‘expanded’ T4SSs have not yet been analyzed by single-particle EM. However, recent advances in *in situ*
 83 cryoelectron microscopy (CryoET) have enabled visualization of the *L. pneumophila* Dot/Icm, *H. pylori* Cag, and
 84 F plasmid-encoded Tra T4SSs (hereafter designated T4SS_{Dot/Icm}, etc.) in the native context of the cell envelope
 85 (14-21). Remarkably, in contrast to the IMC of the VirB₃₋₁₀ structure, the IMCs of all three of the ‘expanded’
 86 systems clearly exhibit 6-fold symmetry and the VirB4 ATPases instead assemble as a central hexamer of dimers
 87 at the channel entrance (16-18).

88

89 In the present study, we solved the *in situ* structure of the pKM101-encoded T4SS (hereafter T4SS_{pKM101}), which
 90 is phylogenetically (**Fig. S1A**) and functionally closely related to the R388-encoded T4SS (T4SS_{R388}) to the extent
 91 that the two ‘minimized’ systems translocate each other’s plasmids and some machine subunits are swappable (8).
 92 We report that the IMC of the *in situ* T4SS_{pKM101} adopts the 6-fold system observed for the equivalent regions of
 93 the ‘expanded’ T4SSs. Most strikingly, the VirB4 homolog is arranged as a central hexamer of dimers, not the
 94 side-by-side hexameric barrels visualized for this ATPase in the purified VirB₃₋₁₀ complex. Our findings support a
 95 unified structural model for the IMCs of T4SSs regardless of their classification as ‘minimized’ or ‘expanded’: in
 96 their native membrane environments these subassemblies are highly symmetric and the signature VirB4 ATPases
 97 invariably adopt a hexamer of dimer configuration at the T4SS channel entrances.

98

99 **Results and Discussion**

100 ***In situ* detection of the pKM101 nanomachine.** To visualize T4SS_{pKM101} nanomachines, we deployed an *E. coli*
 101 *mreB minC* mutant carrying pKM101 to generate small (<300 nm in diameter) minicells (17). Minicells are ideal
 102 for *in situ* CryoET because of their small size and full metabolic capacity, including the capacity to function as
 103 donors of the pKM101 plasmid to recipient cells (**Fig. S1B**) (22). We used a high-throughput CryoET pipeline to
 104 visualize thousands of *E. coli* minicells (see **Fig. S2** for workflow). The pKM101 nanomachines were smaller and
 105 more difficult to detect than the F plasmid-encoded T4SS or other ‘expanded’ systems, but we were able to detect
 106 ~1 or 2 pKM101-encoded structures for every 2 or 3 minicells examined (**Figs. 1Ai, iii, Movie S1**). Importantly,

minicell preparations from the parental strain UU2834 alone lacked these surface structures, confirming that the presence of pKM101 is required for their elaboration.

The pKM101-encoded structures characteristically consisted of periplasmic cone-shaped complexes alone or in association with thin ‘stalk-like structures that extended to the IM (**Fig. S2**). Although the former represented about half of the initially ‘picked’ structures, they were discarded as possible assembly intermediates or dead-end complexes (**Fig. S2**). The pKM101 transfer system also elaborates conjugative pili, and accordingly we detected some pKM101 pili on the surfaces of minicells. Interestingly, these pili were bound either to the presumptive T4SS_{pKM101} machines or to sites on the OM that lacked any underlying basal structures (**Figs. 1Ai, ii, S3**). The pKM101 pili were rarely detected, however, likely because pKM101 and related IncN plasmids elaborate rigid, brittle pili that are readily detached or sloughed from cells (23). Consequently, here we focused on solving the structures of T4SS_{pKM101} machines provisionally composed of the previously-described OMCC and IMC subassemblies (6).

Visualization of the *in situ* OMCC_{pKM101}. From 287 nanomachine subtomograms extracted from 560 tomographic reconstructions, we generated *in situ* structure of the OMCC_{pKM101} at a resolution of ~37 Å (**Figs. 1B, S2**). Three-dimensional classifications revealed 14-fold symmetrical features of the OMCC which were resolved further by imposing a 14-fold symmetry during refinement (**Fig. S2**). In the refined structure, the OMCC is clearly seen attached to the OM where it causes an invagination of the outer leaflet (**Figs. 1Bi, ii**). The upper region, designated as the O-layer in accordance with a nomenclature used to describe the *in vitro* OMCC_{pKM101} structure (4), is 185 Å wide and 70 Å in height. In side-view, the complex forms at least two contacts with the OM, the first mediated by a central cap and the second by the periphery of the OMCC (**Fig. 1Bii**). In the middle of the central cap and extending across the OM is a region of lower density that might correspond to the OM-spanning portion of the translocation channel. In top-down view, the periphery of the OMCC clearly consists of 14 knobs arranged in a ring of ~185 Å (**Fig. 1Biii**). The knobs are connected via spokes to a central contiguous ring that conforms to the base of the cap (**Fig. 1Biii**). In the 3D renderings, it is evident that the 14 peripheral knobs interact with the OM (**Fig. 1C**). Notably, besides invagination of the OM at the cap junction, the region of

the OM delimited by the cap and OMCC peripheral contacts lacks an inner leaflet, suggestive of extensive remodeling of the OM during machine biogenesis (**Figs. 1Bii, iii**).

The chamber within the O-layer widens to ~ 150 Å at the junction with the lower region of the OMCC, which we termed the I-layer again in accordance with a previous nomenclature (4). The I-layer has a height of 65 Å and is slightly wider than the O-layer, although the outer boundary of the O-layer is blurred because of density contributed by the peptidoglycan (PG) layer. The I-layer narrows at its base, and at this position the central chamber has a diameter of ~ 70 Å. A ‘stalk-like’ density embeds into the central cavity and projects through the periplasm to the IM (**Fig. 2B, D**). Combined, the O- and I-layers yield a total height of 135 Å for the *in situ* OMCC structure (**Fig. 2B, D**).

Although the resolution of the visualized OMCC (~ 37 Å) is lower than that achieved by purification and single particle analyses of the corresponding complexes (< 20 Å) (4, 24), the *in situ* and *in vitro* structures are highly similar (**Fig. S4**). Specifically, they exhibit 14-fold symmetry and have cross-section dimensions of 185 Å, and they are composed of distinct O- and I-layers that house large central chambers (4, 7). One difference of interest is that the *in vitro* structures are more elongated (~ 185 Å in height) than the *in situ* structure (135 Å) (**Fig. S4B, C**). An X-ray structure generated for the O-layer of the OMCC (7) fits well into the O-layer of the *in situ* structure (**Fig. S4D**), suggesting that this region of the OMCC purifies well without major distortions. Similarly, the upper portion of the I-layer, which is composed of large, N-terminal globular domains of TraO_{B9}, also has been shown to be intrinsically stable (24). By contrast, the lower portion of the I-layer is built from α -helical linker domains of TraF_{B10}, which connect the OMCC to the IM (24). Although these linker domains were modeled in the *in vitro* structures (4, 24), we suspect they are too flexible for detection *in vivo* or they fold inward to generate part of the central stalk. Accordingly, the OMCC visualized *in situ* would consist of the O-layer and only the upper portion of the I-layer to yield an overall height of 135 Å.

The pKM101-encoded OMCC visualized *in situ* also fitted well on the equivalent subassembly of the VirB₃₋₁₀ complex obtained from the T4SS_{R388} machine (**Fig. S4Ei - iii**), in agreement with earlier findings that the

161 T4SS_{pKM101} retains function when its OMCC is swapped with that from T4SS_{R388} (8). Also of importance, the
 162 thin, central stalk visualized in the VirB₃₋₁₀ complex is also present in the *in situ* T4SS_{pKM101} structure (**Fig. S4Ei-**
 163 **iii**). This confirms that the central stalk is a feature of ‘minimized’ systems assembled *in vivo*, and not a structural
 164 artefact resulting from detergent solubilization and complex purification. As shown with the VirB₃₋₁₀ complex, the
 165 central stalk of the *in situ* T4SS_{pKM101} terminates in the I-layer and forms the only detectable density linking the
 166 OMCC with the IMC. Although, this gap is ~33 Å in the VirB₃₋₁₀ structure, we observed the distance between the
 167 OMCC and IMC of the *in situ* T4SS_{pKM101} machines to be more variable and in the range of ~50 - 70 Å. Perhaps
 168 most importantly, the central stalk lacks a detectable channel (**Figs. 1, S4Eiii**). This distinguishes the ‘minimized’
 169 pKM101 and R388 systems from ‘expanded’ systems such as the F plasmid Tra and *L. pneumophila* Dot/Icm
 170 T4SSs, whose structures were recently solved by *in situ* CryoET (16, 17). In these latter systems, the OMCCs are
 171 invariably connected to their respective IMCs by large cylinders with clearly detectable central channels (**Fig.**
 172 **S4Fi-iii**).

173
 174 **Visualization of the *in situ* IMC_{pKM101}.** Next, we refined the structure of the IMC using class averages in which
 175 machines with clearly discernible OMCC and IMC densities were detected (**Figs. 2A, B, S2**). Notable features of
 176 the IMC include a distinct collar surrounding the central stalk, which in top-down view presents as six knobs
 177 arranged in a ring of 155 Å. The collar was also flanked by six protrusions or ‘bumps’ extending from the IM into
 178 the periplasm (**Fig. 2B, D**). At the cytoplasmic face of the IM, the IMC was dominated by two side-by-side
 179 inverted ‘V’ structures with apices embedded into the IM and ‘arms’ projecting ~80 Å into the cytoplasm (**Fig.**
 180 **2B**). In end-on view, six V structures clearly form two concentric circles, the outer arms of the V’s configured as
 181 a knobbed ring of ~225 Å in diameter and the inner arms joining together as a central hexameric ring with an
 182 outer diameter of ~60 Å and a lumen of ~45 Å. As with the periplasmic collar, the 6-fold symmetry of the
 183 concentric rings was readily visible among the class average images without symmetry imposed (**Fig. S2**). The
 184 structure was resolved further by imposing a 6-fold symmetry during refinement (**Figs. 2B-D, S2**).

185
 186 The structure of the IMC_{pKM101} bears striking similarities to IMCs associated with the ‘expanded’ F plasmid-
 187 encoded Tra and *L. pneumophila* Dot/Icm systems whose structures also were solved by *in situ* CryoET (**Fig. 3A-**

188 C) (16, 17). Most notably, the cytoplasmic complexes of all three systems appear as two inverted V's in side view
 189 and as outer knobbed and inner contiguous rings in end-on view. The outer and inner rings are similar in cross-
 190 section (~225-300 nm) and height (~80 Å), suggestive of a common subunit composition. Supporting this
 191 proposal, structural analyses of mutant machines deleted of each of the T4SS ATPases, coupled with density
 192 tracing of a GFP moiety fused to a VirB4 homolog in the F system, strongly indicate that the inverted V structures
 193 of the F and Dot/Icm systems correspond to dimers of VirB4-like ATPases (16, 17). The cytoplasmic complexes
 194 of the F plasmid and Dot/Icm systems are therefore composed of VirB4 subunits arranged as a hexamer of dimers
 195 surrounding a central channel. The IMC of the *H. pylori* Cag T4SS is architecturally more complex than the IMCs
 196 of the F plasmid-encoded and Dot/Icm systems, yet VirB4-like Cagβ also arranges as a central hexamer of dimers
 197 at the entrance to the Cag translocation channel (18).

198
 199 The IMC_{pKM101} visualized *in situ* differs remarkably from the IMC of the isolated VirB₃₋₁₀ structure (**Figs. 2B, C,**
 200 **3A, 3Di-iii**) (6). In fact, in side view, the two complexes appear generally similar in the presence of side-by-side
 201 densities projecting into the cytoplasm. In end-on view, however, the collar of the *in situ* structure displays 6-fold
 202 symmetry, whereas the corresponding IM platform of the VirB₃₋₁₀ structure is highly asymmetric. The inverted
 203 V's visualized in the *in situ* structure form two concentric rings each with 6-fold symmetry, whereas the
 204 cytoplasmic complex of the *in vitro* structure is arranged as side-by-side barrel complexes (6). Each barrel
 205 comprises three tiers, and a central channel evident in the lower two tiers appears to be closed in the upper tier. By
 206 gold labeling, the barrels were shown to consist at least partly of the VirB4 homolog TrwK (6). A hexameric
 207 arrangement for the two barrels was inferred by earlier findings that TrwK assembles *in vitro* as a homo-hexamer,
 208 and results of stoichiometric analyses showing that the VirB₃₋₁₀ complex is composed of 12 copies of TrwK_{B4} (6,
 209 25).

210
 211 **The pKM101 cytoplasmic complex is dominated by VirB4-like TraB.** To test a prediction that VirB4-like
 212 TraB assembles as a hexamer of dimers at the entrance to the T4SS_{pKM101} channel, we imaged $\Delta traB$ mutant
 213 machines (100 machines from 254 tomographic reconstructions) (**Fig. S5**). *trans* expression of *traB_{B4}* fully
 214 complements the $\Delta traB$ mutation, confirming that the mutation is nonpolar on downstream gene expression (8).

215 All subvolume class averages of the $\Delta traB$ mutant machines showed the prominent OMCC but absence of
 216 discernible IM-associated densities (**Fig. S5**). Most notably, the $\Delta traB$ mutant machines lacked cytoplasmic
 217 densities dominated by the V structures (**Fig. 4B, S5**). The N-terminal domains (NTDs) of VirB4 ATPases
 218 associate tightly with the IM (26, 27), whereas C-terminal domains (CTDs) are highly conserved and adopt a
 219 RecA-like α/β structural fold implicated in ATP binding (28, 29). An atomic structure of the CTD of a VirB4
 220 homolog fitted optimally within densities comprising the proximal halves of the V arms (**Fig. 4Eiii**). This is line
 221 with a proposal that the cytoplasmic complex of the *in situ* T4SS_{pKM101} is composed of TraB_{B4} configured as a
 222 hexamer of dimers, reminiscent of the architectures of the VirB4 homologs in the ‘expanded’ systems (16-18).
 223
 224 Deletion of TraB_{B4} did not affect assembly of the OMCC (**Fig. 4B**), but abolished other IMC densities including
 225 the periplasmic stalk and surrounding collar and ‘bumps’ (**Figs. 4B, S5**). VirB4 homologs associate peripherally
 226 with the IM (30) or at most possess small periplasmic domains (26), arguing against appreciable contributions of
 227 TraB_{B4} to the observed collar and stalk densities. Among the IMC subunits, the linker domains of TraF_{B10} and
 228 VirB5-like TraC_{B5} likely comprise the central stalk as suggested by compositional analyses of the VirB₃₋₁₀
 229 substructure (6). VirB8-like TraE also is a strong candidate for the central collar on the basis of recent nsEM
 230 studies showing that TraE_{B8} assembles as a homohexamer with dimensions matching those of the collar visualized
 231 *in situ* (31). An atomic structure of the periplasmic domain of TraE_{B8} from pKM101 (32) also fits well within
 232 each lobe of the collar (**Fig. 4Eii**). Finally, the polytopic VirB6 subunits typically carry large central periplasmic
 233 domains (33), which might form the ‘bumps’ or also contribute to the central collar or stalk domains. TraB_{B4}
 234 thus might need to be in place in order to recruit the IMC interaction partners to build out the IMC. In line with
 235 this proposal, among various characterized T4SSs, VirB4 subunits have been shown to form extensive networks
 236 of stabilizing interactions with VirB5, VirB8 and VirB10 homologs (8, 34-37).
 237
 238 **Deletions of VirD4-like TraJ or VirB11-like TraG do not detectably alter the *in situ* T4SS_{pKM101}.** Nearly all
 239 T4SSs require a VirD4-like ATPase to couple secretion substrates with the transfer channel. Designated as type
 240 IV coupling proteins (T4CPs) or substrate receptors, VirD4 subunits are members of the SpoIIIE/FtsK
 241 superfamily of motor translocases (38, 39). To determine if VirD4-like TraJ contributes to densities of the

242 T4SS_{pKM101}, we imaged $\Delta traJ$ mutant machines (183 machines from 430 tomographic reconstructions). As
 243 observed with the WT machines, subvolume averaging yielded classes of $\Delta traJ$ mutant machines exhibiting only
 244 the OMCC or both the OMCC and IMC densities (**Fig. S5**). A refined structure generated from the latter showed
 245 no distortions compared to WT machines, insofar as the OMCC, periplasmic collar and central stalk, and
 246 cytoplasmic V structures were clearly evident (**Fig. 4C**). The IMC thus clearly lacks detectable densities
 247 attributable to TraJ_{D4}, which is reminiscent of findings for the F plasmid-encoded Tra and *L. pneumophila*
 248 Dot/Icm systems (16, 17). In the VirB₃₋₁₀ structure, densities thought to correspond to one or two dimers of the
 249 VirD4 homolog were shown to embed between the VirB4 barrels (13). However, our *in situ* findings suggest the
 250 visualized densities likely represent transition-state structures or are artefacts resulting from machine purification.
 251 Evidence that VirD4-like subunits assemble and function as homohehexamers (38, 40), and engage with T4SS
 252 channels only when activated by intracellular signals such as substrate engagement and ATP hydrolysis (41-45)
 253 further supports the notion that the T4CPs engage only transiently with the T4SS channel and therefore are
 254 difficult to detect by *in situ* CryoET.

255

256 Many T4SSs also require a third, VirB11-like ATPase for substrate transfer and pilus production (35, 46). VirB11
 257 ATPases assemble as homohehexamers that fractionate with the cytoplasm and IM, the latter presumably in
 258 association with the T4SS (16, 47-49). To determine if TraG_{B11} contributes to the visualized IMC_{pKM101}, we
 259 imaged $\Delta traG_{B11}$ mutant machines (257 machines from 537 tomograms). Of particular interest, in contrast to the
 260 WT and $\Delta traJ_{D4}$ mutant machines in which IMC densities were detected in only ~50 % of the subvolume class
 261 averages, the fraction of $\Delta traG$ mutant machines with IMCs increased to 85 %. Furthermore, the visualized IMC
 262 densities were more clearly defined than in the class averages for the WT and $\Delta traJ_{D4}$ mutant machines; in
 263 particular, the central stalks were considerably elongated (**Figs. 4, S5**). These findings suggest that TraG_{B11} might
 264 dynamically regulate assembly or conformational status of the central stalk and IMC as a function of ATP
 265 hydrolysis. This proposal is in line with previous work suggesting that VirB11 ATPases regulate the switch
 266 between two alternative structural modalities of T4SSs, the pilus biogenesis factory vs the active translocation
 267 channel (50). We also were unable to detect any density losses in the refined structure of the $\Delta traG$ mutant
 268 machine compared with the WT machine, further suggesting that TraG_{B11} regulates machine biogenesis through

transient interactions with the T4SS_{pKM101} (**Fig. 4**). Consistent with this model, in the *L. pneumophila* Dot/Icm system, VirB11-like DotB shuttles dynamically between the cytoplasm and T4SS_{Dot/Icm} as a function of ATP hydrolysis (16). When engaged with the translocation channel, DotB_{B11} presents as a disc-shaped density attached to the inner hexameric ring of DotO_{B4}. However, in order to visualize this density, it was necessary to deploy a DotB_{B11} mutant that bound but failed to hydrolyze ATP (16).

Summary. CryoET has emerged as a critical complementary approach to single-particle CryoEM studies of bacterial secretion nanomachines (51). Although current resolutions achievable with CryoET are lower than CryoEM, structural definition of machines in their native contexts enables i) validation of architectural features observed *in vitro*, ii) assessments of machine structural variability within and between species, and iii) visualization of dynamic aspects of machine biogenesis and function (3, 51, 52). Here, we presented the first *in situ* structures of a ‘minimized’ T4SS, elaborated by the model conjugative plasmid pKM101. We showed that the T4SS_{pKM101} assembles *in vivo* as two large substructures, the OMCC and IMC, and that the former resembles structures of equivalent complexes solved *in vitro* (4, 7, 24). We further identified specific OM contacts and supplied evidence for OM remodeling during T4SS_{pKM101} biogenesis, and we visualized a central stalk similar to that detected in the isolated VirB₃₋₁₀ complex (6). This latter finding establishes that the central stalk is an important *in vivo* structural element of ‘minimized’ T4SSs and was not a structural artefact of the isolated VirB₃₋₁₀ complex resulting from detergent solubilization. Notably, the central stalk associated with the ‘minimized’ systems lacks a discernible central channel and thus is quite distinct from the corresponding structures connecting OMCCs and IMCs of the ‘expanded’ systems that are configured as larger cylinders with well-defined channels (**Fig. S4F**) (16-18).

Most importantly, we showed that the IMC of the *in situ* T4SS_{pKM101} nanomachine adopts an architecture completely different from that of the isolated VirB₃₋₁₀ structure, especially with respect to the arrangement of the VirB4 ATPase (6). Rather than side-by-side hexameric barrels, VirB4-like TraB assembles as a central hexamer of dimers, an oligomeric state highly reminiscent of those of VirB4 homologs associated with the F-encoded Tra, *L. pneumophila* Dot/Icm and *H. pylori* Cag T4SSs (16-18). To reconcile these different results, we note that, in

contrast to OMCCs, IMCs are characteristically highly unstable and difficult to purify in the presence of detergents, despite use of epitope-tagged subunits during purification such as VirB10-like subunits that stably associate with both subassemblies (4, 9-12, 24). In this context, it is reasonable to suggest that the VirB₃₋₁₀ complex, and particularly its IMC, underwent structural distortions when stripped from the native membrane environment, which would render this portion of the reported structure largely an experimental artefact. Of course, it is feasible that the VirB₃₋₁₀ complexes (6, 13) represent transition-state intermediates associated with machine assembly, but this seems unlikely since we were unable to detect such complexes *in situ*.

Taken together with previous biochemical and structural data (21, 34, 35, 43, 53), our findings support an overarching model highlighting contributions of the VirB4 ATPases to biogenesis of T4SSs regardless of their classification as ‘minimized’ and ‘expanded’ (**Fig. S6**). Assembly initiates by formation of the intrinsically-stable OMCC (**stage I**), which for the T4SS_{pKM101} system can be visualized *in situ* in the absence of associated densities. The OMCC then recruits VirB4 (**stage II**), most likely through previously-identified interactions between the ATPase and the cell-envelope-spanning VirB10 subunit (35). Once positioned, VirB4 recruits other components of the IMC, including subunits such as VirB5, VirB6, and VirB8 that likely contribute to the visualized periplasmic collar and flanking ‘bumps’, and central stalk structures. Upon receipt of an unknown signal, VirB4 then recruits the spatially-dynamic VirB11 ATPase (**stage III**), which induces structural changes in the stalk and IMC of postulated importance for the pilus to channel transition and channel opening (21, 50). Finally, the T4SS channel is activated by a combination of extracellular (target cell contact) and intracellular (substrate docking, T4CP engagement with the T4SS channel, ATP energy utilization) signals, resulting ultimately in the passage of substrates through the VirB4 hexamer and into the envelope-spanning channel (**stage IV**). While there has been remarkable recent progress in defining the architectures of T4SSs, a central question remains how the VirD4 substrate receptor associates with the T4SS and conveys substrates into the channel. Given that this is likely a transient interaction, further deployment of *in situ* CryoET approaches aimed at capturing structural snapshots of T4SSs - including those in the act of translocating substrates - will provide valuable new insights into structural transitions accompanying machine activation.

323 **Materials and Methods**

324 **Strains and growth conditions.** Bacterial strains, plasmids, and oligonucleotides used in this study are listed in
325 Table S1. *E. coli* strains were grown at 37°C in Luria Bertani (LB) agar or broth supplemented with appropriate
326 antibiotics (kanamycin, 100 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹; gentamycin, 10 µg ml⁻¹). Minicells from *E. coli*
327 strain UU2834 were used for all of the CryoET studies.

328
329 **Conjugation assays.** *E. coli* MG1655 strains carrying pKM101 or mutant variants were used as donors to
330 transfer the plasmids into UU2834 recipients. Strains containing the pKM101 mutants also harbored a
331 complementing plasmid. Overnight cultures of donor and recipient cells grown in presence of the appropriate
332 antibiotics at 37 °C were diluted 1:1,000 in fresh LB media, and incubated with shaking for 1.5 h. When needed,
333 cells were induced with arabinose (0.2 % final concentration) and incubated with shaking for another 1.5 h. Equal
334 volumes (50 µl) of donor and recipient cell cultures were mixed and incubated for 3 h at 37 °C. Mating mixtures
335 were serially diluted and plated onto LB agar containing antibiotics selective for transconjugants (Tc's). Plasmid-
336 carrying UU2834 strains were verified for the presence or absence of *tra* genes of interest by PCR, and used for
337 CryoET. For matings to assess minicell donor capacity, minicells were spotted onto a nitrocellulose filter disc
338 alone or with MC4100*rif*^r recipient cells, and the mating mixes were incubated at 37°C for 1 h. The disc was
339 suspended in LB, serially diluted and plated on LB agar plates containing appropriate antibiotics selective for Tc's
340 and donors (to confirm absence of viable donor cells).

341
342 **Isolation of minicells.** *E. coli* minicells were enriched essentially as described previously (54). *E. coli* UU2834
343 harboring pKM101 or variants were grown overnight at 37°C in LB in the presence of spectinomycin, and then
344 subcultured (1:100) in fresh LB devoid of antibiotics at 37°C to an OD₆₀₀=0.5. Anucleate minicells were
345 selectively enriched by centrifugation at 1,000 *x g* for 3 min, the supernatant was centrifuged again at 1,000 *x g*
346 for 3 min, and minicells were passed through a 0.45 µm filter. Minicells were then used in mating assays. To
347 minimize possible breakage of the pKM101-encoded pilus and to concentrate minicells for CryoET analyses,
348 UU2834 strains were grown overnight on LB agar plates at 37 °C. Cells were gently scraped from the plate
349 surface with an “L” shaped rod and resuspended in phosphate-buffered saline (PBS). The cell suspension was

centrifuged twice at 1,000 $\times$ g for 3 min to remove intact cells and the supernatant was then centrifuged at 20,000 $\times$ g for 20 min and the minicell pellet was resuspended in PBS for preparation of grids for CryoET.

Preparation of frozen-hydrated specimens. Minicells resuspended in PBS were mixed with 10 nm diameter colloidal gold particles and deposited onto freshly glow-discharged, holey carbon grids for 1 min. After blotting the grids with filter paper, they were rapidly frozen in liquid ethane by using a gravity-driven plunger apparatus (55, 56).

Cryo-ET data collection and 3D reconstructions. Frozen-hydrated specimens were imaged and data were processed using our previously established protocols (12, 28, 41). Briefly, specimens were subjected to imaging at -170°C using a Polara G2 electron microscope (FEI Company) equipped with a field emission gun and a direct detection device (Gatan K2 Summit). The microscope was operated at 300 kV at a magnification of 15K, resulting in an effective pixel size of 2.5 Å at the specimen level as previously described (17). Tomographic package SerialEM (57) was used to collect low-dose, single-axis tilt series with dose fractionation mode with defocus at about 6 μ m and a cumulative dose of ~ 60 e⁻/Å² distributed over 35 stacks. Each stack contains ~ 8 images. Each tilt series was collected at angles from -51° to 51° with 3° fixed increment. We used Tomoauto (55) to expedite data processing, which included drift correction of dose-fractionated data using Motioncorr (58) and assembly of corrected sums into tilt series, automatic fiducial seed model generation, alignment and contrast transfer function correction of tilt series by IMOD (59), and reconstruction of tilt series into tomograms by Tomo3D (60). Each tomographic reconstruction was 3,710 by 3,838 by 2,400 pixels and ~ 130 Gb in size.

Subtomogram averaging and correspondence analysis. Tomographic package I3 (61) was used for subtomogram analysis as described previously (62). A total of 837 T4SS_{pKM101} machines (400 $\times$ 400 $\times$ 400 voxels) were visually identified and then extracted from 1781 cryo-tomographic reconstructions. Two of the three Euler angles of each T4SS_{pKM101} machine were estimated based on the orientation of each particle in the cell envelope. To accelerate image analysis, 4 $\times$ 4 $\times$ 4 binned subtomograms (100 $\times$ 100 $\times$ 100 voxels) were used for initial alignment. The alignment proceeded iteratively, with each iteration consisting of three parts in which references

and classification masks are generated, subtomograms are aligned and classified, and, finally, class averages are aligned to each other. At the initial iterations, classification mask was applied to include the whole machine and non-T4SS particles were sorted out and removed. For analysis of the IMC, a mask was applied to the IMC only, thus the T4SS particles that did not show IMC density were sorted out and the data set showing IMC was used to further refine the IMC. Classification focusing on the OMC displayed 14-fold symmetry; therefore, 14-fold symmetry was imposed in the following processing to assist the initial alignment process. Furthermore, classification focusing on the IMC showed a 6-fold symmetry feature, and in the following processing 6-fold symmetry was imposed to assist in sub-tomograms alignment. After multiple cycles of alignment and classification for 4×4×4 binned sub-tomograms, 2×2×2 binned sub-tomograms was used for refinement. Fourier shell correlation (FSC) between the two independent reconstructions was used to estimate the resolution of the averaged structures (see Fig. S2).

3D visualization. IMOD was used to visualize the maps and generate 3D surface rendering of *E. coli* minicells. UCSF Chimera (63) (<http://www.rbvi.ucsf.edu/chimera>) was used to visualize subtomogram averages in 3D and for molecular modeling. The video clips for the supplemental videos were made by using UCSF Chimera and further edited with iMovie.

Data availability. Density maps and coordinate data of the T4SS_{PKM101} machines determined by cryo-electron tomography have been deposited in the Electron Microscopy Data Bank (EMDB) as EMD-24100 and EMD-24098. The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information files.

402 LEGEND FOR SUPPLEMENTARY MATERIALS.

403 **Table S1.** List of strains, plasmids, and oligonucleotides used in these studies.

404 **Fig. S1.** Genes and encoded functions of "minimized" type IV secretion systems (T4SSs) in Gram-negative
405 species.

406 **Fig. S2.** Workflow for *in situ* CryoET.

407 **Fig. S3.** Detection of pKM101 pilus docked on *E. coli* outer membrane.

408 **Fig. S4.** Comparisons of the outer membrane core complexes (OMCCs) and stalks/cylinders of 'minimized'
409 and 'expanded' T4SSs.

410 **Fig. S5.** Heterogeneity of the pKM101 T4SS machines revealed by class sub-volume averaging.

411 **Fig. S6.** A unified model for T4SS assembly and routing of secretion substrates through central channels.

412 **Movie S1.** 3-D visualization of a tomographic reconstruction and the T4SS_{pKM101} in *E. coli* minicells.

413 **Movie S2.** 3-D visualization of the T4SS_{pKM101} showing architectural features of the OMCC and IMC and its
414 comparison with purified VirB₃₋₁₀ complex from R388.

415

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420

421 FIGURE LEGENDS

422 **Fig. 1.** *E. coli* minicells carrying pKM101 encoded type IV secretion system (T4SS_{pKM101}) and *in situ* structure of
423 the outer membrane core complex (OMCC) of T4SS_{pKM101} revealed by CryoET and subtomogram averaging. (A i,
424 ii, iii) Tomographic slices from representative *E. coli* minicells showing T4SSs embedded between the outer
425 membrane (OM) and inner membrane (IM). pKM101 pili were associated with a few visualized T4SSs (Ai),
426 although pilus associated OM structures without any periplasmic densities. The T4SS and novel structures are
427 marked with yellow arrow. The boxed regions was magnified to show T4SS with and without associated pilus and
428 also pilus associated OM structures. (A iv) A 3D surface view of the *E. coli* minicell in A iii showing T4SSs. (B i)
429 A central slice of the averaged structure of the T4SS in the cell envelope. (B ii) After refinement, details of the
430 OMCC are visible. The widths and heights of O-layer and I-layer chambers are shown. (B iii) A cross-section
431 view of the region in B ii marked by a yellow arrow shows 14-fold symmetry of the OMCC. (C) 3D surface
432 renderings of the OMCC is shown in different views.

433
434 **Fig. 2.** *In situ* structure of the inner membrane complex (IMC) of pKM101 encoded type IV secretion system
435 (T4SS_{pKM101}) revealed by CryoET and subtomogram averaging. (A) A central slice of the averaged structure of the
436 T4SS in the cell envelope. (B) After refinement, details of the IMC are visible. The stalk, collar and inverted “V”
437 structures along with the height of its arms are shown. (C) Cross-section views of the regions in B marked by
438 yellow arrows show 6-fold symmetry of the IMC. The collar exist as a hexameric ring like structure around the
439 central stalk. (D) 3D surface renderings of the IMC is shown in different views.

440
441 **Fig. 3.** Comparison of the inner membrane complexes (IMCs) solved by CryoET and single particle analysis. (A,
442 B, C) Comparison of the CryoET solved IMCs of type IV secretion systems encoded by pKM101 (T4SS_{pKM101}), F
443 plasmid (T4SS_{pED208}) and *Legionella pneumophila* (T4SS_{Dot/Icm}). 3D surface renderings showing 6-fold symmetric
444 IMCs marked by the hexamer of dimer arrangement of VirB₄ homologs. (D i) A central slice of the averaged
445 structure of the purified VirB₃₋₁₀ encoded by plasmid R388. (D ii) A cross-section view of the region in D i
446 marked by a yellow arrow shows 2-fold symmetry exist in IMC of purified VirB₃₋₁₀. (D iii) The surface rendering

of the VirB₃₋₁₀ substructure highlighting the IMC with side-by-side two hexamers of the TrwK/VirB4 ATPase (pink-shaded). F plasmid EMD: 9344 and 9347; Dot/Icm EMD: 7611 and 7612; VirB₃₋₁₀ EMD:2567

Fig. 4. Architecture of the T4SS_{pKM101} machines from various ATPase mutants and comparison of the structure of the inner membrane complex (IMC) of pKM101 encoded type IV secretion system (T4SS_{pKM101}) with solved structures. (A, B, C, D) The top row shows a central slice of the averaged structure of the T4SS seen in wildtype (WT), $\Delta traB$, $\Delta traJ$ and $\Delta traG$ mutant cells. The second row shows the refined IMCs of the corresponding strains. (A) Native T4SS_{pKM101} machines (WT) for comparisons. (B) The $\Delta traB$ mutant machine lacks the periplasmic collar and cytoplasmic inverted “V” structures. (C, J) Mutant machines lacking TraJ and TraG align well with the WT structure. (E i) 3D surface renderings of the IMC of T4SS_{pKM101} is shown. (E ii) The collar exist as a hexameric ring in the periplasm just above the inner membrane (IM). Periplasmic domain of TraE_{B8} from pKM101 (PDB: 5I97) fits well in the lobe like structure of the collar. (E iii) Fitting of the C-terminal domain (CTD) of VirB4 ATPase from *Thermoanaerobacter pseudethanolicus* (PDB:4AG5) in one of the arms of hexamer of dimers of TraB.

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Fig. 4