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Structural and functional diversity of type IV secretion systems

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19 **Abstract**

20 Considerable progress has been made in recent years in the structural and molecular biology of type IV secretion
21 systems (T4SSs) in Gram-negative bacteria. The latest advances have substantially improved our understanding
22 of the mechanisms underlying the recruitment and delivery of DNA and protein substrates to the extracellular
23 environment or target cells. In this Review, we aim to summarize these exciting structural and molecular biology
24 findings, and to discuss their functional implications for substrate recognition, recruitment and translocation as
25 well as the biogenesis of extracellular pili. We also describe adaptations necessary for deploying a breadth of
26 processes, such as bacterial survival, host–pathogen interactions, and biotic and abiotic adhesion. We highlight
27 the functional and structural diversity of this extremely versatile secretion superfamily to function under
28 different environmental conditions and in different bacterial species. Additionally, we emphasise the
29 importance of further understanding the mechanism of type IV secretion, which will support us in combating
30 antimicrobial resistance and treating T4SS-related infections.

31 [H1] Introduction

32 Type IV secretion systems (T4SSs) are a family of highly complex and versatile nanomachines that span the entire
33 cell envelopes of Gram-positive and Gram-negative bacteria as well as Archaea ¹⁻³ (Figure 1). They function in
34 two main capacities, as DNA transfer (conjugation) systems or as protein effector translocators^{4,5}, generally
35 mediating transfer of macromolecules by mechanisms requiring direct donor–target cell contact. A few systems
36 have evolved the capacity to export DNA or protein substrates to the extracellular milieu, or to take up DNA
37 from it. Many T4SSs also elaborate surface organelles such as conjugative pili or surface adhesins to promote
38 attachment and biofilm formation, but there are also examples of T4SSs that seem to have lost the capacity to
39 translocate substrates and instead function only in adhesion. Considering this enormous range of biological
40 activities, the T4SSs are exceptionally important from a medical perspective. Accordingly, they are increasingly
41 viewed as viable targets for therapeutic intervention to thwart the spread of conjugation-driven antibiotic
42 resistance and infection by pathogens ^{6,7}.

43

44 T4SSs in Gram-negative species are composed minimally of 12 core subunits that are generically termed VirB1 -
45 VirB11 and VirD4 (Ref. ⁸). Systems assembled only with the core VirB–VirD4 components are considered
46 ‘minimized’, and many of these systems function as conjugation machines by delivering DNA substrates to target
47 bacteria^{9,10}. Over the course of evolution, T4SSs have acquired several additional protein components that are
48 integrated into the core structure composed of VirB and VirD4 proteins. As a result, assembly of an expanded
49 T4SS may require up to 25 different proteins ^{10,11}. Some of these expanded systems can mediate conjugative
50 DNA transfer, but many have acquired new functionalities relating to translocation of effector proteins or toxins,
51 with or without retention of the ancestral DNA transfer function^{12,13}.

52

53 In recent years, our understanding of the architectures and mechanisms of actions of T4SSs has increased
54 substantially, most notably by implementation of state-of-the art microscopy techniques including cryo-electron
55 microscopy (cryo–EM), cryo-electron tomography (cryo–ET) and fluorescence microscopy. These studies have
56 enabled visualization of major machine subassemblies and conjugative pili at or near atomic resolution or fully
57 intact T4SSs in the native context of the bacterial cell envelope at lower resolutions (Supplementary Table 1).
58 Most exciting, a very recent study defined for the first time the architecture of a nearly completely intact
59 minimised T4SS encoded by the conjugative plasmid R388 (Ref. ¹⁴). There also has been considerable progress
60 in defining the architecture and mechanisms of action of the VirD4 components of T4SSs; these ATPases have
61 crucial roles in recruiting and coupling substrates to the translocation channel and hence are termed type IV
62 coupling proteins (T4CPs). Finally, studies applying a combination of *in situ* cryo-ET and fluorescence microscopy
63 have advanced our knowledge of T4SS assembly dynamics and spatial organization within intact cells.

64

65 This Review will primarily focus on the T4SSs found in Gram-negative bacteria, as they have been the most
66 extensively characterized in terms of both structure and function. We will summarise the current knowledge of
67 paradigmatic T4SSs functioning in these bacteria with a focus on their architectures and adaptations for
68 specialised functions. We also update the reader on recent studies exploring the biogenesis pathways and spatial

69 localization of T4SSs, and we conclude with a brief review of progress toward developing small molecule
70 inhibitors of T4SSs and manipulating these versatile nanomachines for novel therapeutic ends.

71

72 [H1] Architectures of minimised systems

73 Early biochemical studies supplied evidence that the VirB subunits VirB7, VirB9 and VirB10 assemble as a
74 stabilizing structural scaffold for the T4SS; this scaffold ultimately was designated as the outer membrane core
75 complex (OMCC)^{8,15}. OMCCs are intrinsically stable and amenable to isolation for structural characterization.
76 Accordingly, over a decade ago, high-resolution structures were presented for the OMCC associated with a
77 minimized T4SS elaborated by the conjugative plasmid pKM101 (T4SS_{pKM101})^{16,17}, and soon afterwards for several
78 other OMCCs from minimized systems¹⁸⁻²¹. The most recent structure presented for the nearly intact T4SS
79 encoded by plasmid R388 (T4SS_{R388}) now has provided important refinements of these earlier structures¹⁴
80 (Figure 2a). The OMCC from the T4SS_{R388} presents as a barrel-shaped structure of 130 Å in height and 185 Å in
81 width. It is composed of an outer and inner layer that are designated as the O-layer and I-layer, respectively.
82 Remarkably, in contrast to the lower-resolution structures obtained previously, the new structure shows that
83 the O- and I-layers have different symmetries (Figure 2a). The O-layer is made up of 14 copies of homologs of
84 the VirB7 lipoprotein and C-terminal domains (CTDs) of the VirB9- and VirB10-like subunits. VirB7 anchors the
85 OMCC to the outer membrane via its N-terminal lipid modification. The I-layer assumes a 16-fold symmetry,
86 formed by copies of N-terminal domains (NTDs) of the VirB9 and VirB10 subunits. The C14:C16 symmetry
87 mismatch between the O- and I-layers is accommodated by a unique configuration of two VirB9 and VirB10
88 subunits; while their NTDs insert in the I-layer, their CTDs do not form part of the O-layer¹⁴ (Figure 2a).
89 Importantly, the VirB10_{CTD} of the O-layer contains a hydrophobic 2-helix bundle (termed the antennae
90 projection) extending from the VirB10_{CTD} β-barrel domains, and 14 of these bundles assemble to form a hollow
91 pore through the outer membrane. This pore has a diameter of 32 Å at the extracellular entrance, which is
92 proposed to expand further through a hinge-like conformational change between the antennae projection
93 helices¹⁴ to accommodate a growing pilus and substrate translocation. This mode of action is in line with earlier
94 findings that VirB10 associated with the *Agrobacterium tumefaciens* VirB–VirD4 T4SS (T4SS_{Agro}) undergoes a
95 conformational switch in response to sensing and transduction of intracellular signals to gate the outer
96 membrane channel²²⁻²⁵.

97

98 The phytopathogen *Xanthomonas citri* elaborates a T4SS whose constituent subunits are close homologs of
99 components of the pKM101- and R388-encoded conjugation machines, but this system (T4SS_{X. citri}) functions as
100 an interbacterial killing machine by delivering toxins to neighbouring bacteria²¹. Interestingly, the OMCC of the
101 T4SS_{X. citri} does not display a mismatch between the O- and I- layers as both assume a 14-fold symmetry²¹. It
102 encodes a much larger VirB7 homologue with a pronounced C-terminal N0 domain²⁶, a feature also found in
103 the Dot/Icm system (T4SS_{Dot/Icm}) encoded by *Legionella pneumophila* (see below)^{27,28}. The N0 domain widens
104 horizontally, giving rise to a flying saucer-shaped structure rather than the barrel-like structures of OMCCs from
105 the T4SS_{pKM101} and T4SS_{R388} machines. Whether or how these structural variations contribute to the specialized
106 function of the T4SS_{X. citri} machine in interbacterial transmission of toxins is not yet known.

107

108 The nearly intact T4SS_{R388} also details the organization of the inner membrane complex (IMC) and periplasmic
109 stalk (Figure 2a). The stalk connecting the OMCC and IMC has an overall diameter of ~92 Å and length of 216 Å.
110 The stalk consists of a pentamer of VirB5 subunits located proximally to the OMCC connected to a pentamer of
111 VirB6 that inserts into the inner membrane by an N-proximal α-helix¹⁴ (Figure 2a). Six knobs (or ‘arches’)
112 composed of the C-terminal domains of VirB8 subunits surround the central stalk near the inner membrane. The
113 architecture of the stalk is intriguing in view of prior evidence that the VirB5 subunit localizes at the tip of the T-
114 pilus elaborated by the *A. tumefaciens* VirB/VirD4 system²⁹. Moreover, the finding that the bulk of VirB6 resides
115 in the periplasm completely revises our views of how VirB6 contributes to substrate translocation and pilus
116 assembly. VirB6 subunits are highly hydrophobic, and previously were envisioned to adopt a polytopic topology
117 in the inner membrane and form part of the inner membrane channel^{1,30}. In the T4SS_{R388} structure, the bulk of
118 VirB6 is shown to assemble as a pentameric platform entirely in the periplasm while only one N-terminal
119 hydrophobic domain made of two trans-membrane helices integrates in the inner membrane (Figure 2a). Thus,
120 most of the hydrophobic domains of VirB6 subunits are shielded from the aqueous environment of the periplasm
121 through extensive intersubunit contacts. It is also noteworthy that the pentameric symmetry of the VirB5–VirB6
122 stalk matches that of the conjugative pilus.

123

124 Using structural-complementarity simulations, the VirB2 pilin component of the conjugative pilus was
125 successfully docked on the VirB6 platform, which led to a new model for how conjugative pili assemble (Figure
126 2b). Upon synthesis, pilin subunits integrate into the inner membrane, forming a pool for subsequent use in
127 building the pilus³¹ (Figure 2b, step 1). Co-evolution studies confirmed by site-directed mutagenesis identified
128 one of the two trans-membrane helices as binding and recruitment site for the VirB2 pilin. Upon receipt of an
129 unknown signal, five pilin subunits are extracted from the inner membrane into the periplasmic assembly site
130 located in the interacting surfaces of the VirB6 and VirB5 proteins (Figure 2b, step 2). Through reiterative
131 recruitment and extraction of pilins to the VirB6 platform, the pilus extends and displaces the VirB5 pentamer
132 upwards and through the outer membrane to the extracellular milieu¹⁴ (Figure 2b, step 3). The VirB5 pentamer
133 remains at the tip of the pilus, where it binds specific receptors on the target cell surface or embeds directly into
134 the target cell membrane³² (Figure 2b, step 4). The role of VirB5 as an adhesin was initially speculated in 2008
135 (Ref. ³¹), and subsequent evidence has been found in the Cag system. In this system, the functional orthologue
136 of VirB5, termed CagL³³⁻³⁵, has been shown to bind integrin on the human cell surface³². Furthermore, VirB5 has
137 exhibited conformational changes similar to those observed in pore-forming proteins¹³, suggesting potential
138 structural parallels with the translocon pores of type III secretion systems.

139

140 The IMC of the T4SS_{R388} is dominated by two concentric rings that extend into the cytoplasm (Figure 2a). These
141 rings comprise a hexamer of dimers of the VirB4 ATPase¹⁴. The dimers are arranged so that one protomer forms
142 the inner ring and the other forms the outer ring. Protomers of each dimer are connected by their NTDs, which
143 also embed into the inner membrane. Additionally, the NTDs of protomers comprising the inner and outer
144 hexameric rings form specific contacts with the VirB3 and VirB8 subunits, respectively. This interaction network

145 anchors the VirB4 hexamer of dimers in the inner membrane and potentially enables ATP-dependent structural
146 changes ¹⁴ (Figure 2a).

147

148 Structures of the T4SS_{pKM101} and of several mutant machines assembled within the cell envelope also were solved
149 by *in situ* cryo-ET ³⁶ (Figure 2a). Despite their lower resolutions, these structures revealed several distinctive
150 features. For example, during assembly of the T4SS_{pKM101}, the outer membrane is extensively remodelled as
151 evidenced by invagination of the outer leaflet and the absence of the inner leaflet of the OM at the machine -
152 OM junction. The OMCC possesses a central chamber sufficiently large to accommodate a growing pilus as it
153 extends from the stalk assembly platform. Most importantly, analyses of the IMCs from WT and mutant strains
154 established that the VirB4 subunit assembles *in vivo* as a central hexamer of dimers at the channel entrance,
155 and that neither of the VirB11 or VirD4 ATPases contribute to IMC densities ³⁶. These latter ATPases also were
156 not visualized in the *in vitro* T4SS_{R388} structure, but the VirB11–VirB4 interaction was characterised using
157 computational methods and validated biochemically and by site-directed mutagenesis. VirB11 and VirD4 might
158 dock transiently with the VirB channel in response to unknown signals ¹³.

159

160

161 [H1] Architectures of expanded systems

162 Expanded T4SSs are composed of up to 25 distinct proteins, encompassing the 12 core components of VirB and
163 VirD4 along with additional protein subunits ^{10,11}. The most widely studied expanded systems are the F plasmid-
164 encoded conjugative machine, and the *L. pneumophila* Dot/Icm and *Helicobacter pylori* Cag effector translocator
165 systems. Although detailed structures for the IMCs from these systems remain to be determined, near atomic
166 resolution structures are now available for OMCCs from all three systems, F plasmid-encoded conjugative pili,
167 and the T4SS_{Dot/Icm} substrate recognition platform.

168

169 The OMCC associated with the F system is composed of the three core VirB components, VirB7-like TraV, VirB9-
170 like TraK and VirB10-like TraB ^{15,37} (Figures 3a and 3b). It adopts a ‘flying saucer’ shape and is much larger than
171 the OMCCs of the minimized conjugation machines, with a diameter of ~268 Å and width of 115 Å. The complex
172 consists of concentric rings termed the outer and inner rings. The inner ring exhibits a 17-fold symmetry and is
173 composed of seventeen CTDs of TraB and NTDs of TraV. The outer ring displays a 13-fold symmetry and is
174 composed of 26 CTDs of TraV and 26 copies of TraK (Figure 3a). The TraK subunits assemble as 13 dimers, with
175 the CTDs of each dimer pair forming 13 elongated knobs that extend radially from the centre of the complex.
176 The TraB and TraV proteins were observed to form flexible linkers connecting the symmetrically mismatched
177 inner ring and outer ring. Structural flexibility imparted by the symmetry mismatch could account for the
178 dynamic properties of the F pilus during extension and retraction. This ability could also be potentiated by a
179 number of additional proteins present in the expanded F system compared to minimised conjugative systems,
180 which are not capable of pilus retraction.

181

182 The *L. pneumophila* Dot/Icm and *H. pylori* Cag systems function as effector translocators that aid in infection
183 processes (Figure 1). The Dot/Icm system has the remarkable capacity to translocate at least 330 effector
184 proteins, nearly 10 % of the proteome, into eukaryotic cells^{28,38,39}. Effector translocation induces a myriad of
185 physiological changes marked by conversion of human phagosomes into replication-permissive compartments
186 called *Legionella*-containing vacuoles (LCV)⁴⁰. The OMCC_{Dot/Icm} has a highly complex organisation and is
187 considerably larger (~420 Å in diameter) than that of the F system (Figure 3a). VirB7-like DotD, VirB9-like DotH,
188 and VirB10-like DotG proteins form the structural scaffold, which is built upon by incorporation of other Dot/Icm-
189 specific proteins including DotF, DotC, Dis1, Dis2 and Dis3 (Refs. ^{41,42}). The OMCC_{Dot/Icm} has a three-layer topology
190 with a disk-shaped outer membrane cap (OMC) of 13-fold symmetry, a proximal dome of 16-fold symmetry and
191 a smaller periplasmic ring of 18-fold symmetry (Figure 3a). Distinct O- and I- layers reminiscent of the OMCC_F
192 are likely to be represented by the OMC and PR domains, respectively. In line with this proposal, VirB9-like DotH
193 was observed to comprise both the OMC and periplasmic ring reminiscent of the architectures of the VirB9
194 counterparts in the F, pKM101 and R388 systems.

195

196 The Cag system of *H. pylori* secretes the oncoprotein CagA as well as several other nonproteinaceous substrates
197 into human epithelial cells⁴³⁻⁴⁵ (Figure 1). These T4SS_{Cag}-mediated functions induce pathological changes in
198 epithelial cells that facilitate *H. pylori* infection of the gastrointestinal tract⁴⁶. The T4SS_{Dot/Icm} and T4SS_{Cag} are the
199 largest of the T4SSs characterized to date and both possesses some of the structural properties identified in the
200 other systems. The mushroom-shaped OMCC_{Cag} is 400 Å in width and 250 Å in height, and is composed VirB7-
201 like CagT, VirB9-like CagX and VirB10-like CagY plus two system-specific proteins, Cag3 and CagM⁴⁷ (Figure 3a).
202 The large size of the OMCC_{Cag} is conveyed by the larger sizes of CagT, CagX and CagY relative to their VirB
203 counterparts, and by incorporation of multiple copies of Cag3 and CagM in the structure. The OMCC_{Cag} consists
204 of an OMC with clear O- and I-layers. The OMC is made up of CagT, CagX, CagY, Cag3 and CagM, and the
205 periplasmic ring composed of CagY and CagX. Portions of the periplasmic ring show structural resemblance to
206 the I-layer of OMCC_{*X. citri*} (Ref. ⁴⁸). Both the inner and outer regions of the OMC have a 14-fold symmetry, whereas
207 the periplasmic ring assumes a 17-fold symmetry (Figure 3a). As shown for other VirB9 subunits, CagX forms
208 contacts with both the OMC and periplasmic ring, thus bridging the symmetry mismatch between those two
209 complexes⁴⁸. Reminiscent of the F system, the observed asymmetries among OMCC substructures of the *L.*
210 *pneumophila* Dot/Icm and *H. pylori* Cag systems might have evolved to confer specialized functions, in these
211 cases relating specifically to the infection process. OMCC structural flexibility might, for example, be needed to
212 establish dynamic yet productive contacts with eukaryotic host cells or to coordinate the timing and delivery of
213 substrates into the eukaryotic cell targets.

214

215 These expanded T4SSs also have been visualized by *in situ* cryo-ET in their native cellular contexts. Remarkably,
216 F systems elaborate a presumptively quiescent translocation channel and three morphologically distinct
217 platforms upon which the F pilus is docked⁴⁹ (Figure 3b). The channel, designated as the F1-channel complex,
218 consists of the OMCC joined to the IMC by a periplasmic cylinder that is thicker and more pronounced than the
219 thin periplasmic stalk of the T4SS_{R388} (Figure 3b). The central hexamer of dimer configuration of the VirB4-like

220 TraC ATPase is readily visualized at the cytoplasmic entrance, reminiscent of the *in situ* T4SS_{pKM101} and recent *in*
221 *vitro* high resolution T4SS_{R388} structures (Figure 2a). An F-pilus associated structure, termed the F2–channel–
222 pilus complex, resembles the F1 complex but has the F pilus attached at the distal end of the OMCC (Figure 3b).
223 The F1 and F2 complexes are postulated to correspond to the quiescent channel and active pilus-assembly
224 factory involved in mate-seeking and mating. Two other F pilus-associated structures, designated as the F3–talk–
225 pilus and F4–outer membrane-pilus complexes, consist of the F pilus attached respectively to a thin stalk density
226 that spans the periplasm or a small outer membrane density without any underlying structure. The F3 and F4
227 complexes lack discernible channels for substrate transfer or pilus assembly, and accordingly are proposed to
228 function exclusively as holding platforms for nonretractile F pili. These inert structures might contribute to
229 nonspecific cell aggregation and biofilm formation or as decoys for bacteriophages that rely on F pilus retraction
230 to gain access to the cell envelope⁴⁹.

231
232 Visualization of the *in situ* T4SS_{Dot/Icm} structure revealed an OMCC with a ‘Wi-Fi symbol’-like architecture of 400
233 Å in diameter whose assembly is dependent on the DotC, DotD, DotF, DotG and DotH subunits^{50,51} (Figure 3c).
234 The OMCC is connected to a cylinder with an outer diameter of ~20nm and a central lumen or channel of ~6 nm,
235 which narrows to a diameter of ~10nm and a channel of ~3nm near the IM⁵¹. As with other T4SSs (Refs. 14,21,36),
236 at the cytoplasmic face of the IMC is the hexamer of dimer configuration of VirB4 ATPase (DotO). Remarkably,
237 VirB11-like DotB, a second ATPase of this system, assembles as a hexamer that dynamically associates with the
238 DotO inner hexamer by a mechanism dependent on cycles of ATP binding and hydrolysis (see below)⁵¹. Recently,
239 another Dot/Icm system elaborated by *Coxiella burnetii* has been determined by a combination of cryo-ET and
240 cryo-focused ion beam (cryo-FIB) milling to closely resemble that of *L. pneumophila*⁵². Interestingly, this study
241 documented a correlation between assembly of the machinery and developmental transitions of *C. burnetii*
242 cells during infection, which complements previous observations that reported a dependence of effector
243 translocation by this T4SS on the progression of *C. burnetii* infection⁵³.

244
245 In the *in situ* cryo-ET map of the *H. pylori* Cag machine, the OMCC resembles the equivalent substructure solved
246 at a higher resolution by cryo-EM^{48,54-56}. Analyses of mutant machines confirmed that *in situ* assembly of the
247 OMCC requires the Cag subunits CagX, CagY and CagM, and that CagT and Cag3 contribute to peripheral
248 densities. Other noteworthy features of the *in situ* T4SS_{Cag} machine include a hollow cylinder that extends across
249 the periplasm, connecting the OMCC to the IMC. The IMC is architecturally more complex than the equivalent
250 substructures of the F and Dot/Icm systems in having three concentric rings instead of two. A unique feature
251 among T4SSs is that the extracellular domain of the CagY subunit of the T4SS_{Cag} contains multiple binding sites
252 for Toll-like receptor 5 (TLR5) and functions in regulating immune responses of the host⁵⁷. The inner and middle
253 rings correspond to the central and outer hexamers of VirB4-like CagE, but assembly of the outer ring is
254 dependent on production of VirD4-like Cagβ⁵⁵. VirB11-like Cagα associates at the base of the CagE central
255 hexamer, but in contrast to the Dot/Icm system, Cagα seems to associate stably with CagE and not dynamically
256 as a function of ATP binding and hydrolysis as shown for DotB⁵⁵ (Figure 3d). A comprehensive table that

257 summarises all available structures to date comprising both, the minimised and expanded systems is provided
258 as Supplementary Table 1.

259

260

261 **[H1] Other T4SS machine adaptations**

262 Besides appropriating novel subunits for functional diversification, many T4SSs of both the minimised and
263 expanded types have diversified through modifications of core VirB components. VirB2-like pilins or pili or
264 associated surface adhesins have been adapted to enhance T4SS targeting to specific cell types (see below),
265 whereas certain IMC or OMCC components have undergone modifications through acquisition of novel domains
266 for broadened T4SS functionality (Figure 4). These modifications generally function to direct machine assembly
267 at specific sites within the cell or coordinate T4SS localization or function with the cell cycle, or to promote
268 attachment to specific target cells, as described in more detail in supplementary text box 1. Symmetry
269 mismatches are likely to provide regions of mobility or flexibility between different protein complex layers or
270 subassemblies. However, despite the presence of symmetry mismatch in several types of secretion systems,
271 precisely how symmetry mismatch contributes to machine functions at mechanistic or structural levels have not
272 been established for any characterized secretion system ¹⁰.

273

274 The VirB6 subunits are the most extensively modified of the IMC components. In many T4SSs, VirB6 subunits are
275 composed predominantly of 5-7 hydrophobic domains that are likely to assemble as part of the central stalk
276 structure, as demonstrated for the R388-encoded VirB6 subunit (Figure 1a). However, many VirB6 subunits
277 termed 'extended-VirB6s' are considerably larger by virtue of the presence of one or more large central of C-
278 terminal hydrophilic domains¹ (Figure 4). Remarkably, where characterized these hydrophilic domains
279 contribute in distinct ways to establishment or inhibition of productive donor–target cell interactions. In F
280 systems, a large ~600 residue C-terminal hydrophilic domain (CTD) of VirB6-like TraG is involved in entry
281 exclusion, a process that blocks redundant DNA transfer between the donor cells. When donor cells form mating
282 junctions with other donor cells, the CTD of TraG produced by one donor cell establishes contact with TraS_F, an
283 inner membrane protein produced by the paired donor cell ^{58,59}. This contact is achieved either by extension of
284 TraG_F across the mating junction or by proteolytic cleavage and translocation of the CTD of TraG_F through the
285 T4SS_F into the paired donor cell. In *Rickettsia spp.*, multiple copies of extended-VirB6s are present with sizes
286 ranging from 600 to over 1400 residues ^{60,61}. Large hydrophilic domains are surface displayed where they are
287 implicated in promoting endosymbiotic or pathogenic relationships with eukaryotic target cells⁶². In the *L.*
288 *pneumophila* Dot/Icm system, VirB6-like DotA possesses multiple hydrophobic domains flanking a central
289 hydrophilic domain. Although DotA associates with the inner membrane reminiscent of other VirB6 family
290 members, it can also be exported in a Dot/Icm T4SS-dependent manner to the extracellular milieu where it forms
291 ring-like oligomers whose functions are presently unknown ⁶³.

292

293 All three of the core VirB components of OMCCs can be modified through acquisition of novel motifs (Figure 4).

294 Although many VirB7-like lipoproteins are small (~50 residues) and resemble the archetypal *A. tumefaciens*

295 VirB7, many others are considerably larger (~160-300 residues) as shown for *H. pylori* CagT, *X. citri* VirB7 and *L.*
296 *pneumophila* DotD^{11,21,41,48}. In the *H. pylori* system, both CagX and CagY are considerably larger than their VirB9
297 and VirB10 counterparts. Indeed, in the case of CagY, only the extreme C-terminal region adopts the
298 characteristic VirB10 β -barrel-domain folds that assemble as the central rings of OMCCs. A large middle repeat
299 region (MRR) is composed of multiple repeats, which undergo extensive rearrangement during gene expression.
300 These rearrangements yield many CagY variants that have been shown to regulate T4SS_{Cag} function — positively
301 or negatively — to maximize persistent infection⁶⁴⁻⁶⁶.

302

303

304 **[H1] VirD4 substrate recruitment and translocation**

305 For most T4SSs, VirD4-like ATPases also known as type IV coupling proteins (T4CPs)^{22,23,67} are responsible for
306 substrate recruitment. T4CPs characteristically possess an N-terminal transmembrane domain (TMD) implicated
307 in establishment of contacts with IMC components of cognate T4SSs. A conserved nucleotide-binding domain
308 (NBD) is thought to provide the energy for early-stage substrate processing; for example, unfolding and opening
309 of the channel for substrate transfer. Two sequence-variable domains, the all-alpha domain (AAD) and C-
310 terminal domain (CTD) if present, contribute to substrate recruitment. An X-ray structure of R388-encoded
311 TrwB, currently the structural archetype for T4CPs, showed that the NBD assembles as a homohexamer⁶⁸ (Figure
312 5a). This NBD architecture bears similarities to the FtsK and SpoIIIE families of DNA motor proteins involved in
313 DNA translocation during cytokinesis⁶⁷. The AAD sits at the base of the hexamer, optimally positioned for
314 docking of secretion substrates. Indeed, evidence has now been presented for binding of AADs to secretion
315 substrates in the *A. tumefaciens* and *Xanthomonas* VirB–VirD4 systems^{69,70}. Protein substrates are recognised
316 by the T4SS through conserved motifs called translocation signals. These highly specific signals encoded by the
317 protein substrates are tailored for the timely engagement and controlled secretion by the T4S apparatus. Details
318 on the different translocation signals associated with T4SS substrates can be found in the supplementary text
319 box 2.

320

321 VirD4 homologues also interact with VirB11 ATPases^{22,71,72}. VirB11 family members are structurally similar
322 (Figure 5b) insofar as their NTDs and CTDs resemble each other and are connected by a flexible linker. VirB11
323 subunits assemble as homohexamers⁷³, and in this oligomeric state, the interdomain linkers facilitate fluent
324 domain swaps without affecting hexamer assembly⁷⁴. The VirB11, VirD4 and VirB4 ATPases act in concert to
325 orchestrate pilus biogenesis and substrate transfer, although mechanistic details underlying this coordination of
326 ATPase functions remain unknown^{22,71,72,75,76}. The VirB11 ATPases are viable drug targets, as illustrated by an
327 early report that small molecule inhibitors of VirB11-like Cag α blocks virulence of *H. pylori*⁷⁷.

328

329 In the *L. pneumophila* Dot/Icm system, the CTD of VirD4-like DotL has a highly complex role in recruitment of its
330 many protein effectors and their subsequent secretion through the T4SS_{Dot/Icm} apparatus (Figure 5c). Although
331 recruitment of various effectors to DotL can proceed independently of known associated chaperones,
332 recruitment of others is strictly dependent on the IcmS and IcmW chaperones, with or without an additional

333 requirement for the LvgA chaperone⁷⁸⁻⁸¹. DotL assembles as a hexamer, and associated with each of the six CTDs
334 are various Dot adaptors including DotM, DotN, DotY, DotZ, IcmS and IcmW. The DotM adaptor itself also acts
335 as a recruitment platform for the some of the IcmSW-independent effectors⁸². The six CTD-adaptor complexes
336 together form a bell-shaped structure, termed the type IV coupling complex (T4CC), that extends into the
337 cytoplasm and functions as the substrate recruitment platform (Figure 5c). The T4CC possesses at least two
338 known effector binding-sites, one on DotM and a second strictly dependent on the IcmS and IcmW chaperones,
339 with or without an additional requirement for the LvgA chaperone⁷⁸⁻⁸¹. Through binding of distinct arrays of
340 effectors based on their associations with different chaperones, the T4CC is envisioned to regulate substrate
341 transfer during *L. pneumophila* infection⁸³⁻⁸⁸.

342

343 In the *H. pylori* Cag T4SS, VirD4-like Cag β is structurally similar to TrwB (Figure 5a); however its AAD contributes
344 in a unique way to modulation of Cag β function. In this system, two cytosolic proteins, CagF and CagZ, function
345 together with Cag β to recruit the CagA substrate to the T4SS_{cag}. Prior work showed that CagF functions as a
346 chaperone by binding a 100-residue region of CagA, whereas CagZ stabilizes Cag β ⁸⁹. Recently, crystal structures
347 presented for the Cag β AAD–CagZ interaction led to a model whereby the CagZ–AAD contact maintains Cag β in
348 a monomeric state, thereby suppressing ATPase activity and rendering Cag β inactive⁹⁰. Upon receipt of an
349 unknown signal, CagF binds and recruits CagA to Cag β , resulting in release of CagZ and assembly of the Cag β
350 hexamer. This catalytically active form of Cag β then unfolds and translocates CagA through the T4SS_{cag}.

351

352 Among the conjugative T4SSs, T4CPs recruit a specialised DNA-processing complex called the relaxosome^{5,91}
353 (Figure 6a). In the well-characterized F system, the relaxosome is composed of four proteins assembled at the
354 origin-of-transfer (*oriT*) sequence of the F plasmid. The largest relaxosome subunit, Tral relaxase (~200 kDa),
355 possesses a trans-esterase domain that nicks and covalently attaches to the 5' end of the nicked DNA strand
356 destined for translocation (T-strand). Tral also possesses a vestigial helicase domain that serves as a single-
357 stranded DNA (ssDNA)-binding domain, an active 5' to 3' helicase domain, and a C-terminal domain that
358 functions as a recruitment platform for the remaining relaxosome components^{92,93}. Two other relaxosome
359 components TraY and IHF cause conformational changes in the DNA topology that expose the nick (*nic*) site for
360 cleavage by Tral.^{94,95} Finally, TraM is a homotetramer responsible for docking the DNA–relaxosome complex to
361 the coupling protein TraD^{96,97} (Figure 6a). On the DNA side, a pair of TraM tetramers recognise a particular DNA
362 sequence known as *sbmABC* motifs within the *oriT* of the F-plasmid⁹⁸, resulting in up to six tetramers bound to
363 one F plasmid. Each TraM tetramer is subsequently recognised by C-terminal tails of the hexameric TraD⁹⁶ (Figure
364 6a). This interaction ensures F plasmid docking and transfer while actively blocking translocation of a co-existing
365 conjugative plasmid(s) through the F machine^{84,97,98}.

366

367 Remarkably, at this time there is little detailed structural information concerning the physical relationship
368 between the T4CP and the T4SS channel. Consequently, the route or routes by which DNA and protein substrates
369 are conveyed through T4SSs is not known, and two distinct translocation pathways have been envisioned (Figure
370 6). As illustrated for the T4SS_{dot/lcm} machine, upon recruitment of substrates to the DotL T4CC, the T4CC functions

371 in one of two ways, which ultimately dictates whether substrates are delivered in one or two steps across the
372 entire cell envelope (Figure 6B). In the one-step translocation pathway⁹⁹, DotL is situated beneath or near the
373 T4SS and, upon substrate engagement, the T4CC passes captured substrates to the base of the T4SS channel
374 marked by the DotB and DotO hexameric ATPases. The DotL–DotB–DotO ATPase ternary complex then
375 orchestrates substrate unfolding, dissociation of chaperones and/or adaptors, and delivery of the translocation
376 intermediate into the channel for conveyance in one step through a channel that extends from the cytoplasmic
377 face of the inner membrane to the cell exterior (Figure 6b). In the alternative two-step translocation model⁹⁹,
378 DotL is situated in physical proximity to the T4SS. The T4CC captures and then shunts substrates directly into the
379 lumen of the DotL hexamer (whose NTD spans the inner membrane) for delivery across the inner membrane.
380 Once in the periplasm, in a second translocation reaction, substrates are recruited to and enter the T4SS channel
381 for delivery to the cell surface^{83,51,100} (Figure 6b). For reasons outlined in the next paragraph, we hypothesize
382 that delivery of the F plasmid transfer intermediate follows the one-step pathway, whereby TraD docks at the
383 base of the channel in complex with VirB4-like TraC⁸⁴ (Figure 6a).

384

385 In the absence of detailed structural information about the T4CP–T4SS connection, we currently favor the one-
386 step pathway for DNA as well as protein substrates, given the potentially deleterious consequences
387 (degradation, misfolding, misrouting and temporal disruption) encountered by ssDNA transfer intermediates or
388 as many as several hundred effectors delivered into the bacterial periplasm via the two-step pathway. Early
389 crosslinking studies in *A. tumefaciens* system also supplied experimental support for a one-step pathway. In
390 those studies, DNA substrates of the VirB–VirD4 system were shown to engage in sequential order with ATPase
391 subunits comprising the cytoplasm–inner membrane interface, then with components of the IMC, and finally
392 with components of the OMCC and pilus^{22,23,30,71,101}. Structural studies of the T4SS_{R388} by negative stain-EM¹⁰²
393 and of the T4SS_{Cag} by *in situ* cryo-ET⁴⁹ also have supplied evidence that T4CPs can associate with the VirB4
394 hexameric platform at the channel entrance, thus being optimally positioned to deliver captured substrates
395 directly into the channel. While there are clear examples of T4SSs that utilize two-step translocation pathways
396 to deliver substrates to the extracellular milieu¹⁰³ or to eukaryotic target cells¹⁰⁴, these systems lack VirD4 T4CPs
397 and thus rely on alternative secretion systems for recruitment and translocation of substrates across the inner
398 membrane.

399

400

401 [H1] Conjugative pili and target cell attachment

402 T4SSs elaborate various surface structures that have important roles in promoting donor–target cell contacts.
403 Among these, conjugative pili are major contributors to the rapid and widespread dissemination of plasmids and
404 other mobile elements, and their cargoes of antibiotic resistance determinants, among Gram-negative bacteria.
405 High-resolution structures recently have been generated for several conjugative pili, including those produced
406 by F plasmids (F pilus)^{105,106}, the *A. tumefaciens* VirB–VirD4 system (T pilus)¹⁰⁷⁻¹⁰⁹ and IncN plasmid pKM101¹⁰⁸.
407 Throughout evolution, the relationships between hosts and pathogens have led to adaptations in T4SS-
408 associated pili, resulting in more specialized functions. Using a ‘one-size-fits-all’ approach is not effective for

409 adhesion to distinct host-encoded receptors or different biotic and abiotic surfaces. As a result, pili and pilins
410 have diversified to fulfil these functions. The supplementary text box 3 summarizes the diversification of pili and
411 bacterial adhesins and their role in establishing close contacts with target cells.

412

413 Structurally, assembled pili differ in rotational raise of subunits (Figure 7a) and can display either a five-start
414 helical symmetry, as shown for T- and N-pili and F-pili encoded by the classical F plasmid and F-like pED208, or
415 a one-start symmetry as shown for another F-pilus encoded by pKpQIL or an Archaeal conjugative pilus¹⁰⁹. The
416 outer diameters of conjugative pili in Gram-negative species are 76 Å - 87 Å and inner lumens are 23 Å - 26 Å in
417 width, whereas the archaeal pilus has an inner diameter of 16 Å (Figure 7b).

418

419 As noted earlier, pilin subunits accumulate in the inner membrane as a pool for recruitment to build the
420 conjugative pilus upon receipt of an unknown signal. Intriguingly, structural studies of the conjugative pili have
421 established that, during extraction from the inner membrane, pilins co-extract phospholipid molecules. In F
422 systems, for example, TraA pilins co-extract phosphatidylglycerol molecules in a 1:1 stoichiometry (Figure 7C),
423 resulting in assembly of the helical fiber with phosphatidylglycerol molecules lining the pilus lumen. These
424 phosphatidylglycerol molecules impart an overall negative charge to the F pilus lumens^{105,106}. The *A. tumefaciens*
425 T pilus^{108,109} and pKM101-encoded N pilus¹⁰⁸ share the general features first reported for F pili, but the lumens
426 are lined with phospholipids with different head groups (Figure 7c). For N pili this results in an overall negative
427 charge reminiscent of F pili¹⁰⁸, but for T pili the lumen has an overall positive charge. Another interesting
428 difference is the presence of a kink between the first alpha helices of pilins comprising the T- and N- pili, which
429 is absent in pilins associated with pili from expanded systems (Figure 7c; arrow). Why conjugative pili differ in
430 phospholipid compositions and what is the significance of the kink between helices $\alpha 1$ and $\alpha 2$ is unknown, but
431 it is reasonable to propose that these features impart biophysical properties of importance for specialized
432 functions in different environmental conditions.

433

434 In support of this model, recent biophysical studies have demonstrated that F pili are extremely flexible and
435 have spring-like properties with pronounced structural and thermochemical robustness. These properties are
436 postulated to accelerate conjugation rates and biofilm formation by F-plasmid-carrying cells by allowing for
437 effective function even in highly turbulent environments, such as those present in human gastrointestinal tracts
438 ¹¹⁰. This unique feature of F-pili might well be responsible for recent evidence that IncF plasmids are the most
439 dominant types of conjugative plasmids present in enterobacterial isolates from humans and animals ¹¹¹. In
440 contrast to F pili, other conjugative pili are generally shorter and more rigid and have not been shown to
441 retract¹¹². Because of their distinctive biophysical properties, these rigid pili are envisioned to readily break from
442 the cell surface, accumulate in the milieu, and mediate nonspecific aggregation of donor and potential recipient
443 cells, thus acting indirectly to facilitate propagation of mobile elements.

444

445

446 **[H1] Conclusions and outlook**

447 The remarkable recent progress in defining the architectures of T4SSs, both *in vitro* and *in situ*, confirms that the
448 functional diversity of these fascinating nanomachines is recapitulated at the structural level. Although there
449 clearly are emergent structural themes, most if not all T4SSs also have acquired systems-specific properties. This
450 is particularly evident with the expanded systems, which have appropriated components from unknown
451 ancestries that physically enlarge and add structural complexity to the T4SSs. The contribution of T4SS structural
452 diversity to their varied functions is still not fully understood. However, it is possible to speculate on potential
453 relationships. For instance, differences in pilin or adhesin subunits may result in variations in pilus biogenesis,
454 assembly and adherence properties. Another factor to consider is cargo recruitment, where structural
455 differences in the T4CP receptor or complex are likely to have evolved to specify restricted or expanded
456 substrate repertoires. Additionally, T4SS diversity at both the structural and functional levels are likely to have
457 evolved for recognition of specific host cell receptors, ultimately dictating the range of hosts that the T4SS can
458 target. Interestingly, DNA and protein substrates also have evolved a bewildering array of translocation signals
459 (supplementary text box 2), along with deployment of chaperones or adaptors, for docking with VirD4 substrate
460 recruitment platforms. In conjunction, VirD4 evolved to carry sequence-variable motifs or domains (for example,
461 AAD, CTD and T4CC) to specify and temporally regulate loading and delivery of substrates into the translocation
462 channel.

463

464 The T4SS field is well-poised to answer several long-standing questions. Most importantly, we still lack critical
465 information about the physical and functional relationship of the VirD4 T4CP with the cognate T4SS channel.
466 Also, what constitutes the translocation channel across the periplasm and the route of substrate transfer across
467 the entire cell envelope? At the cell exterior, how do mating junctions form, how are they physically configured,
468 and how do they dissociate after substrate transfer is completed? To what extent or for which systems do
469 conjugative pili routinely transmit DNA and protein substrates through their lumens? To address these
470 questions, a combination of structural analyses of mutant machines, fixed in their activated states or actively
471 engaged in substrate transfer, and other approaches such as correlative light and electron microscopy (CLEM)
472 offer considerable promise. Finally, and most central to this Review, how do the various T4SS structural
473 adaptations confer system-specific functions? A full answer to this question will be generated by widening the
474 current subjects of study to include the many other T4SSs functioning in diverse species of bacteria and archaea.

475

476 There is growing interest in translational initiatives aimed at blocking or repurposing T4SSs for therapeutic ends.
477 High-throughput screens are being used to identify small-molecule inhibitors, with goals of blocking conjugative
478 dissemination of antibiotic resistance or inhibiting effector translocation to suppress pathogenesis^{6,7,113-120}.
479 Structural advances of the different T4SSs summarized in this Review will continue to facilitate the rational
480 design of small molecules effective at blocking critical subunit–subunit interfaces. Conversely, as T4SSs are the
481 only bacterial secretion systems capable of delivering DNA or protein substrates to a wide range of target cell
482 types, these nanomachines are excellent delivery systems for therapeutic interventions. Indeed, the early
483 discovery of *A. tumefaciens*-mediated T-DNA transfer, along with the realization that any DNA of interest can be
484 substituted for oncogenic T-DNA, spawned an entirely new field of plant genetic engineering¹²¹. In recent years,

485 conjugation machines have been repurposed to deliver CRISPR–Cas9 systems to bacterial recipients to cure drug
486 resistance plasmids or kill recipient cells harbouring CRISPR–Cas9 target sequences ¹²²⁻¹²⁵. Very recently,
487 bacterial donors engineered to surface display nanobodies were shown to selectively deliver DNA cargoes to
488 recipient cells displaying the cognate antigens ¹²⁶. These types of translational advances set the stage for
489 deployment of T4SSs for selective killing of bacterial targets or even of cancer cells.

490

491 In the 20 years since the state of knowledge of the fascinatingly versatile T4SSs was first reviewed², the field has
492 made astounding progress in defining many T4SS structures and mechanisms of actions, and identifying the
493 range of cellular consequences accompanying effector translocation. We fully expect the next 20 years to yield
494 even more exciting fundamental and translational advances.

495

496

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501

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503 T.R.D.C, J.B.P. and P.J.C. wrote the article. T.R.D.C., J.B.P., P.J.C. and K.M. researched data for the article. K.M.
504 made figures and table with contributions from T.R.D.C. and J.B.P. All authors reviewed and/or edited the
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506

507 **Competing interests**

508 The authors declare no competing interests.

509

510 **Supplementary information**

511 Supplementary information is available for this paper at <https://doi.org/10.1038/s415XX-XXX-XXXX-X>

512

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869

870 **Figure 1. The functional versatility of type IV secretion systems.**

871 Various pathogenic bacteria and symbionts deploy type IV secretion systems (T4SSs) to deliver effector proteins,
872 DNA–protein complexes or other macromolecules into eukaryotic or protozoan host cells. **a** | The T4SSs
873 establishes contact dependent interkingdom interactions by injecting effectors directly into eukaryotic cells to
874 promote bacterial intracellular survival and symbiosis. **b** | Many bacterial species and a few Archaea deploy a
875 contact-dependent T4SSs for the delivery of DNA and toxins to other bacteria or Archaea. Various species in the
876 *Xanthomonadales* instead deploy T4SSs for the contact-dependent delivery of protein toxins to kill other
877 bacteria for niche establishment. **c** | Some bacteria can deploy T4SSs for the contact-independent uptake or
878 release of DNA.

879

880 **Figure 2. Structure of minimal type IV secretion system and pilus biogenesis mechanism model.**

881 **a** | Global organisation to atomic details of the minimised R388-encoded type IV secretion system (T4SS). The
882 entire cryo-electron microscopy (cryo-EM) structure of the R388 T4SS is shown with half-left in cryo-EM density
883 colored by sub-complexes (Electron Microscopy Data Bank (EMDB) entry 12707, EMDB 12708, EMDB 12709,
884 EMDB 13767 and EMDB 12933) and half-right in ribbon and surface semi-transparent representation colored by

885 proteins (Protein Data Bank (PDB) identifier 7O3J, PDB ID 7O3T, PDB ID 7O3V, PDB ID 7Q1V and PDB ID 7OIU).
886 At top-left corner, the cryo-ET of pKM101 T4SS density (EMDB 24098 and 24100) colored by sub-complexes is
887 displayed to show that the structure of purified R388 T4SS is similar to the *in situ* T4SS structure. For each sub-
888 complex, structure details, symmetry and membrane localisation are indicated. Black dashed lines demarcate
889 the boundaries of the outer membrane and inner membrane. **b** | Model of pilus biogenesis mechanism. The
890 T4SS is schematically represented in slice-view and colored by protein. Four states are shown: T4SS in similar
891 state as observed by cryo-EM and shown in part a (1). The pilus biogenesis state with VirB11 bound at the
892 bottom of VirB4; VirB2 is extracted from the inner membrane and recruited to VirB6 through the coordinated
893 actions of the VirB4–VirB11 ATPases (2). As layers of VirB2 are recruited, the pilus grows from the VirB6
894 assembly sites and VirB5 remains at the pilus tip (3). As the pilus grows, the O-layer of the OMCC opens up and
895 the pilus with VirB5 at the tip extends into the extracellular milieu to establish contact with potential recipient
896 cells (4). Part a is adapted with permission from Ref 14.

897

898 **Figure 3. Structural organisation of expanded type IV secretion system.**

899 **a** | Three expanded type IV secretion system (T4SS) outer membrane core complex (OMCC) structures are
900 shown. The OMCC of the F-plasmid (Protein Data Bank (PDB) identifier 7OKN and PDB ID 7OKO), *Legionella*
901 *pneumophila* (PDB ID 7MUS) and *Helicobacter pylori* (PDB ID 6X6S and 6X6J) are shown in surface representation
902 and colored in dark red, blue and green for the VirB7-, VirB9- and VirB10-like proteins, respectively, and in grey
903 for other components. Notably, *L. pneumophila* and *H. pylori* OMCCs contain an outer membrane cap (OMC)
904 and a periplasmic ring (PR). **b** | Cryo-tomography maps of the F-plasmid with and without pilus. The maps
905 (Electron Microscopy Data Bank (EMDB) entry 9344 and 9347) are colored by subcomplexes (that is; green for
906 the OMCC, red for the Stalk, yellow for the Arches, blue for the inner membrane complex (IMC) and grey for the
907 pilus. The junction of the pilus and Stalk is not well defined. **c** | Cryo-electron microscopy (cryo-ET) map of the
908 *L. pneumophila* T4SS (EMDB entry 7611 and 7612) colored as in b, in front and slice view. **d** | Cryo-ET of the *H.*
909 *pylori* T4SS (EMDB entry 0634 and 0635) colored as in b, in front and slice view.

910

911 **Figure 4. Examples of type IV secretion system subunit adaptations for functional diversification.**

912 The R388-encoded type IV secretion system T4SS is shown at left for reference. VirB5 subunits are deployed for
913 binding of target-cell receptors; these subunits can localize at the tips of conjugative pili or on the bacterial cell
914 surface. Some bacteria encode several copies of VirB2 or VirB5 subunits whose variable sequences are
915 postulated to bind different target-cell receptors or contribute to evasion of the host immune system. Extended
916 VirB6 carry large hydrophilic domains, several of which have been shown or are implicated in localizing at the
917 cell surface to promote adhesion or immunomodulation, or blocking redundant plasmid transfer. F systems
918 elaborate F pili that dynamically extend and retract to establish contacts with potential recipient cells at a
919 distance. F systems also code for TraN subunits, whose extracellular domains interact with outer membrane
920 proteins (OMPs) on recipient cells to promote F plasmid transfer and specify plasmid host range. Several T4SSs
921 possess variant forms of the VirB7–VirB9–VirB10 core complex subunits, as exemplified for CagY in the *H. pylori*
922 Cag system. The *H. pylori* system elaborates a conjugative pilus, which is decorated by other Cag subunits as well

923 as the CagA secretion substrate. Various T4SSs functionally interact with other surface adhesins, such as
924 pKM101-encoded Pep or *H. pylori* OMPs, to promote target-cell binding.

925

926 **Figure 5. Structure of VirD4- and VirB11-like ATPases.**

927 **a** | Side and top view of two VirD4-like ATPase structures: TrwB from R388 plasmid (Protein Data Bank (PDB)
928 identifier (ID) 1GKI) and Cag β from *H. pylori* (PDB ID 8DOL). Structures are shown in ribbon representation and
929 colored by monomer. **b** | Side and top view of two VirB11-like ATPase structures; DotB from *L. pneumophila*
930 (PDB ID 6GEF) and Cag α from *H. pylori* (PDB ID 1NLZ). Structures are shown in ribbon representation and colored
931 by monomer. **c** | Organisation and structure of type 4 coupling complex (T4CC) from *L. pneumophila*. The
932 monomeric cryo- electron microscopy (cryo-EM) structure of the T4CC (PDB ID 6SZ9) is shown in surface
933 representation and colored by protein: VirD4-like DotL in red), DotM in cyan, DotN in blue, DotZ in yellow, DotY
934 in green, IcmS in pink and IcmW in purple. The module made up of the extreme C-terminus of (DotL_{C-ter}) and
935 IcmSW is flexible, and its motion area is represented in mesh. Crystal structure of the DotL_{C-ter}–IcmSW module
936 in presence of LvgA adaptor (orange) and VpdB substrate (black) is shown (PDB ID 7BWK). On the right, a model
937 of the hexameric T4CC structure is shown in front and top views using the same color coding as in part c.

938

939 **Figure 6. Models for substrates recruitment and transport through the type IV secretion system.**

940 **a** | Conjugative type IV secretion system (T4SS) recruitment and secretion mechanism model. Silhouette of F-
941 plasmid T4SS is shown in green. First, the DNA is processed by a relaxosomal complex made of TraM (green),
942 TraY (turquoise), IHF (purple) and TraI (light brown). The relaxosome is recruited by TraD_{VirD4} ATPase, which
943 energizes the secretion of the TraI–ssDNA through the T4SS apparatus into the host. The relative position of
944 TraD_{VirD4} and the global organisation of the inner membrane complex (IMC) during DNA secretion is unknown. **b**
945 | T4SS effector recruitment and secretion mechanism model. Silhouette of *L. pneumophila* Dot/Icm T4SS is
946 shown in blue. The type IV coupling complex (T4CC) acts as a effector recruitment platform and is schematically
947 represented and positioned beside the complex formed by the hexameric dimers of DotO and DotB, although
948 its precise localisation is unknown. Effector proteins are captured by the T4CC at different binding sites and
949 DotL_{VirD4} energizes substrate translocation via one of two possible routes across the inner membrane. Route 1:
950 The T4CC feeds substrates into the DotO–DotB energy centre at the base of the T4SS channel for transit in one-
951 step across the entire cell envelope. Route 2: the T4CC feeds substrates into the lumen of the DotL hexamer for
952 delivery across the inner membrane. In a second translocation step, substrates are recruited from the periplasm
953 by the T4SS channel for passage to the cell surface and into target cells. Part b is adapted with permission from
954 Ref. 28.

955

956 **Figure 7. Structure comparison between minimal, expanded and archaea pilus.**

957 **a** | Side view of all known pilus structures. Pilus structures (Protein Data Bank (PDB) identifier (ID) 8EXH
958 *Agrobacterium tumefaciens* T-pilus, PDB ID 8CW4 *Escherichia coli* N-pilus; PDB ID 5LER *E. coli* F-pilus; PDB ID
959 5LEG *Salmonella typhimurium* pED208; PDB ID 7JSV *Klebsiella pneumoniae* pKpQIL; PDB ID 8DFU *Aeropyrum*
960 *pernix* CedA1 and PDB ID 8DFT *Pyrobaculum calidifontis* TedC) are in surface representation with one strand

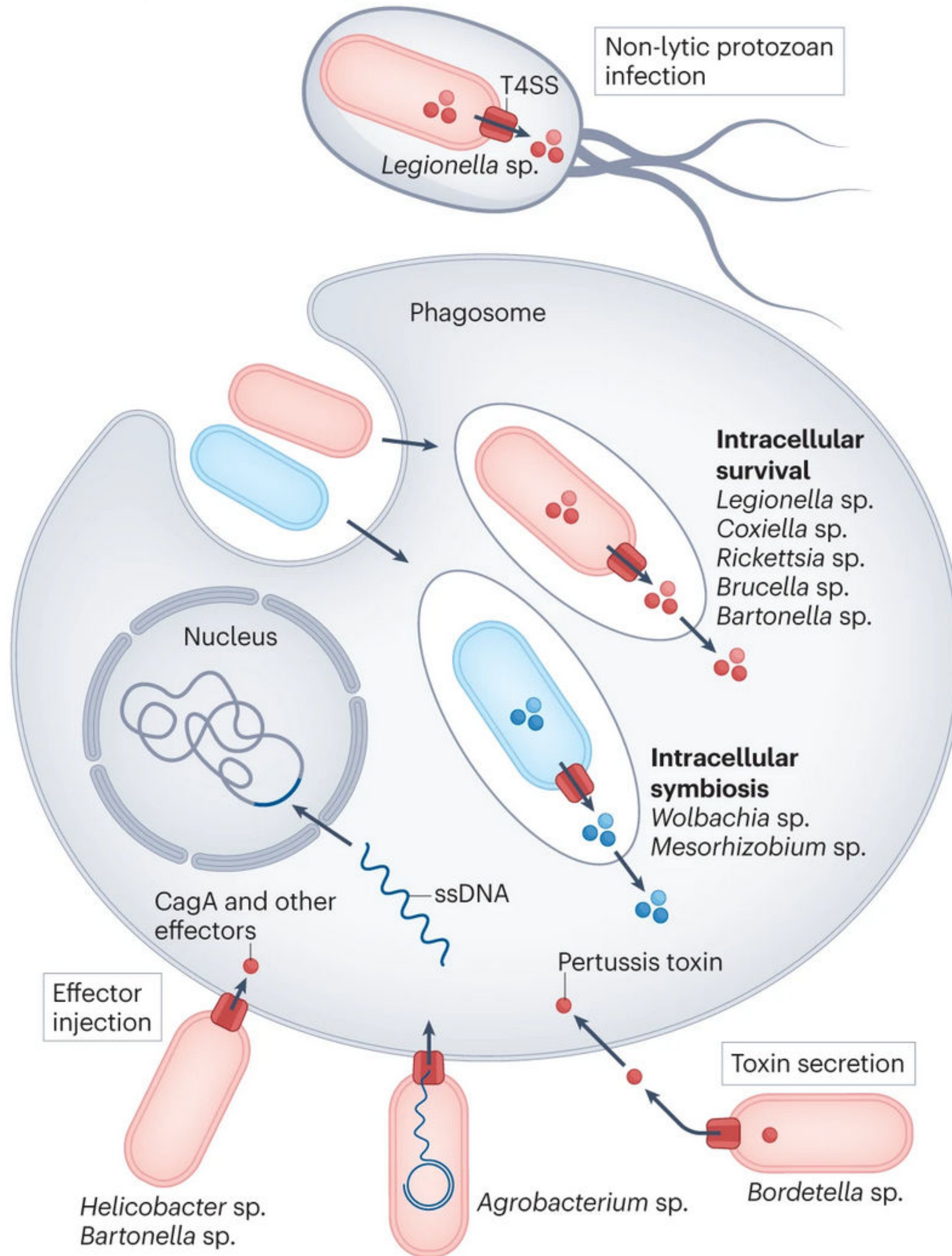
961 colored in grey. **b** | Top view of pilus structures. Diameter and lumen sizes are indicated. **c** | For each pilus, one
962 monomer of VirB2 with its lipid is shown in ribbon representation. Arrows in the minimised type IV secretion
963 systems (T4SSs) highlight the presence of a 'kink', which is characteristic of this group. Parts a and b adapted
964 with permission from Ref. 109.

Fig. 1: The functional versatility of type IV secretion systems.

From: [Structural and functional diversity of type IV secretion systems](#)

a

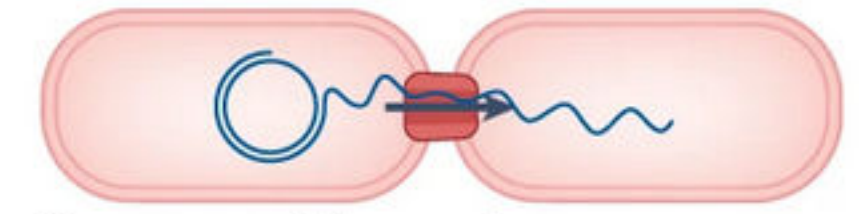
Contact-dependent interkingdom



b

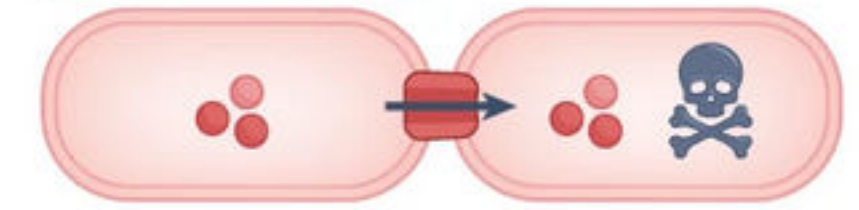
Contact-dependent interbacterial

Conjugation



Gram-positive and Gram-negative bacteria

Toxin delivery

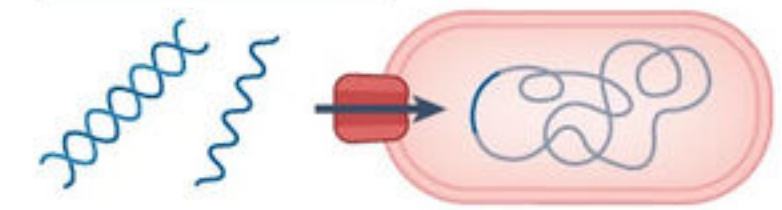


Xanthomonadales

c

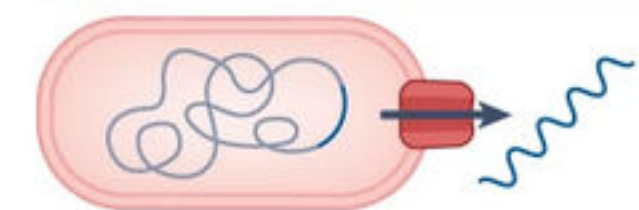
Contact-independent DNA uptake or release

DNA uptake



Helicobacter pylori

DNA release and biofilm formation

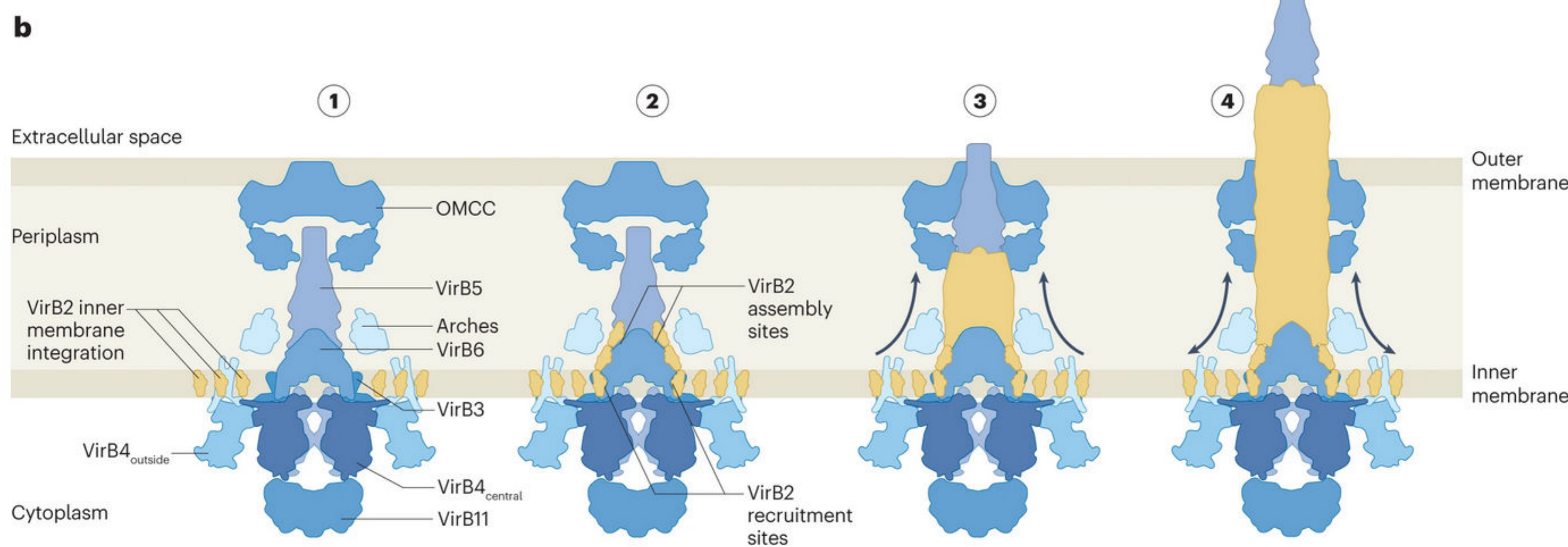
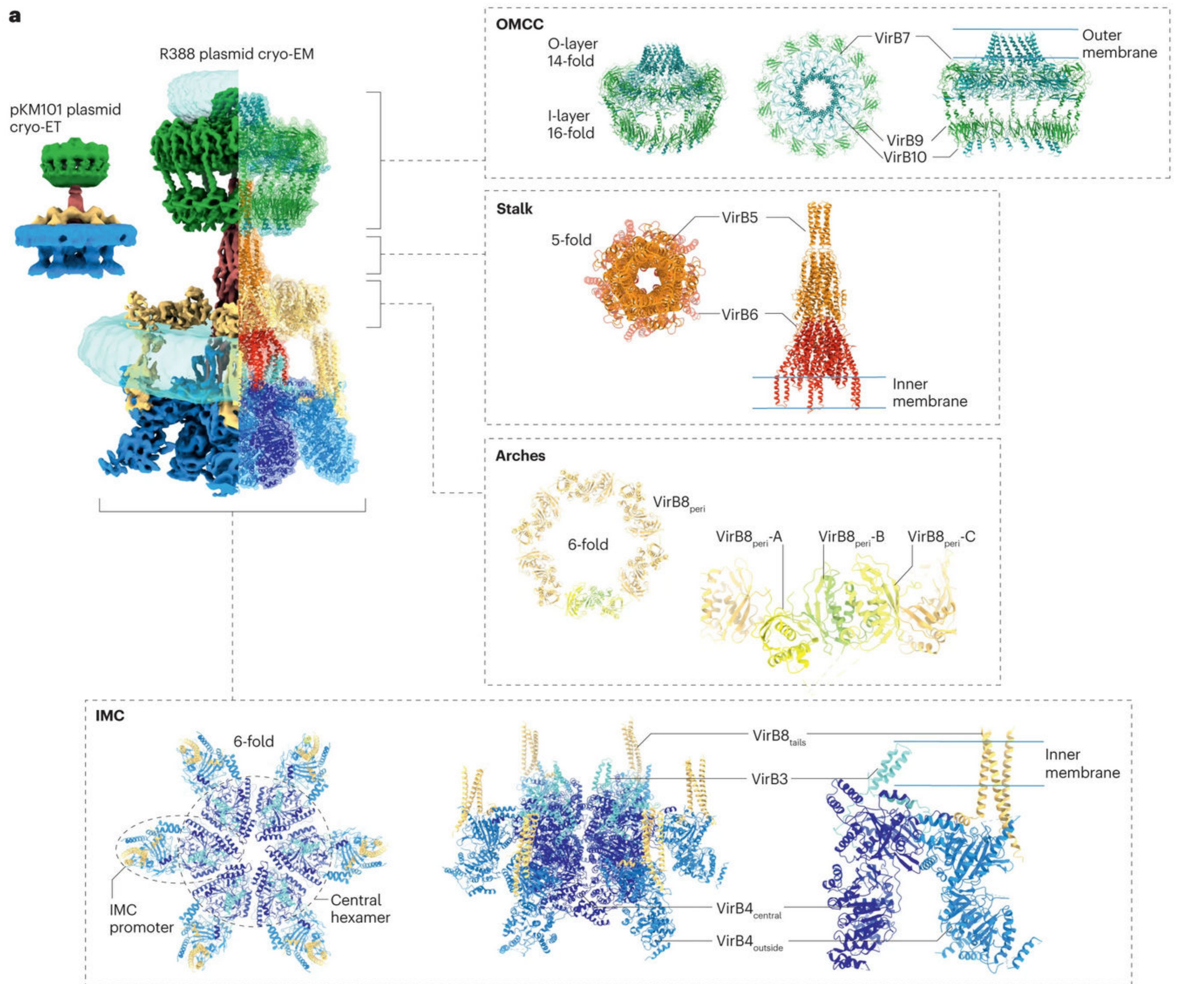


Neisseria gonorrhoeae

Various pathogenic bacteria and symbionts deploy type IV secretion systems (T4SSs) to deliver effector proteins, DNA-protein complexes or other macromolecules into eukaryotic or protozoan host cells. **a**, The T4SS establishes contact-dependent interkingdom interactions by injecting effectors directly into eukaryotic cells to promote bacterial intracellular survival and symbiosis. **b**, Many bacterial species and a few Archaea deploy a contact-dependent T4SS for the delivery of DNA and toxins to other bacteria or Archaea. Various species in the *Xanthomonadales* instead deploy T4SSs for the contact-dependent delivery of protein toxins to kill other bacteria for niche establishment. **c**, Some bacteria can deploy T4SSs for the contact-independent uptake or release of DNA. ssDNA, single-stranded DNA.

Fig. 2: Structure of minimal type IV secretion system and pilus biogenesis mechanism model.

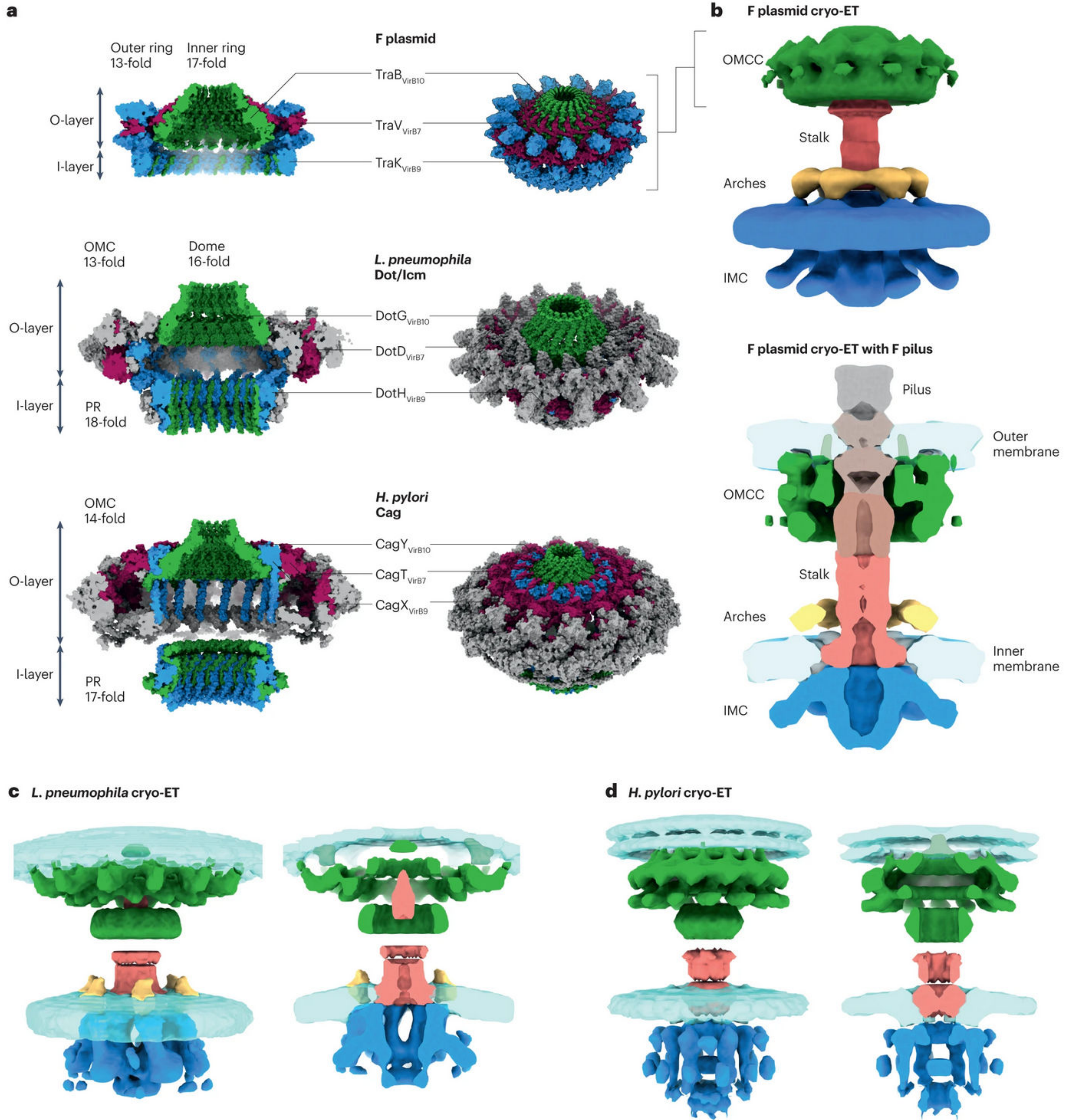
From: [Structural and functional diversity of type IV secretion systems](#)



a, Global organization to atomic details of the minimized R388-encoded type IV secretion system (T4SS). The entire cryo-electron microscopy (cryo-EM) structure of the R388 T4SS is shown with half-left in cryo-EM density coloured by sub-complexes (Electron Microscopy Data Bank (EMDB) entry 12707, EMDB 12708, EMDB 12709, EMDB 13767 and EMDB 12933) and half-right in ribbon and surface semi-transparent representation coloured by proteins (Protein Data Bank (PDB) identifier (ID) 7O3J, PDB ID 7O3T, PDB ID 7O3V, PDB ID 7Q1V and PDB ID 7O1U). In the top-left corner, the cryo-electron tomography (cryo-ET) of pKM101 T4SS density (EMDB 24098 and 24100) coloured by sub-complexes is displayed to show that the structure of purified R388 T4SS is similar to the in situ T4SS structure. For each sub-complex, structure details, symmetry and membrane localization are indicated. Black dashed lines demarcate the boundaries of the outer membrane and inner membrane. **b**, Model of pilus biogenesis mechanism. The T4SS is schematically represented in slice view and coloured by protein. Four states are shown: (1) T4SS in similar state to that observed by cryo-EM and shown in part **a**. (2) The pilus biogenesis state with VirB11 bound at the bottom of VirB4; VirB2 is extracted from the inner membrane and recruited to VirB6 through the coordinated actions of the VirB4–VirB11 ATPases. (3) As layers of VirB2 are recruited, the pilus grows from the VirB6 assembly sites and VirB5 remains at the pilus tip. (4) As the pilus grows, the O-layer of the outer membrane core complex (OMCC) opens up and the pilus with VirB5 at the tip extends into the extracellular milieu to establish contact with potential recipient cells. IMC, inner membrane complex. Part **a** adapted from ref. [14](#), CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

Fig. 3: Structural organization of expanded type IV secretion system.

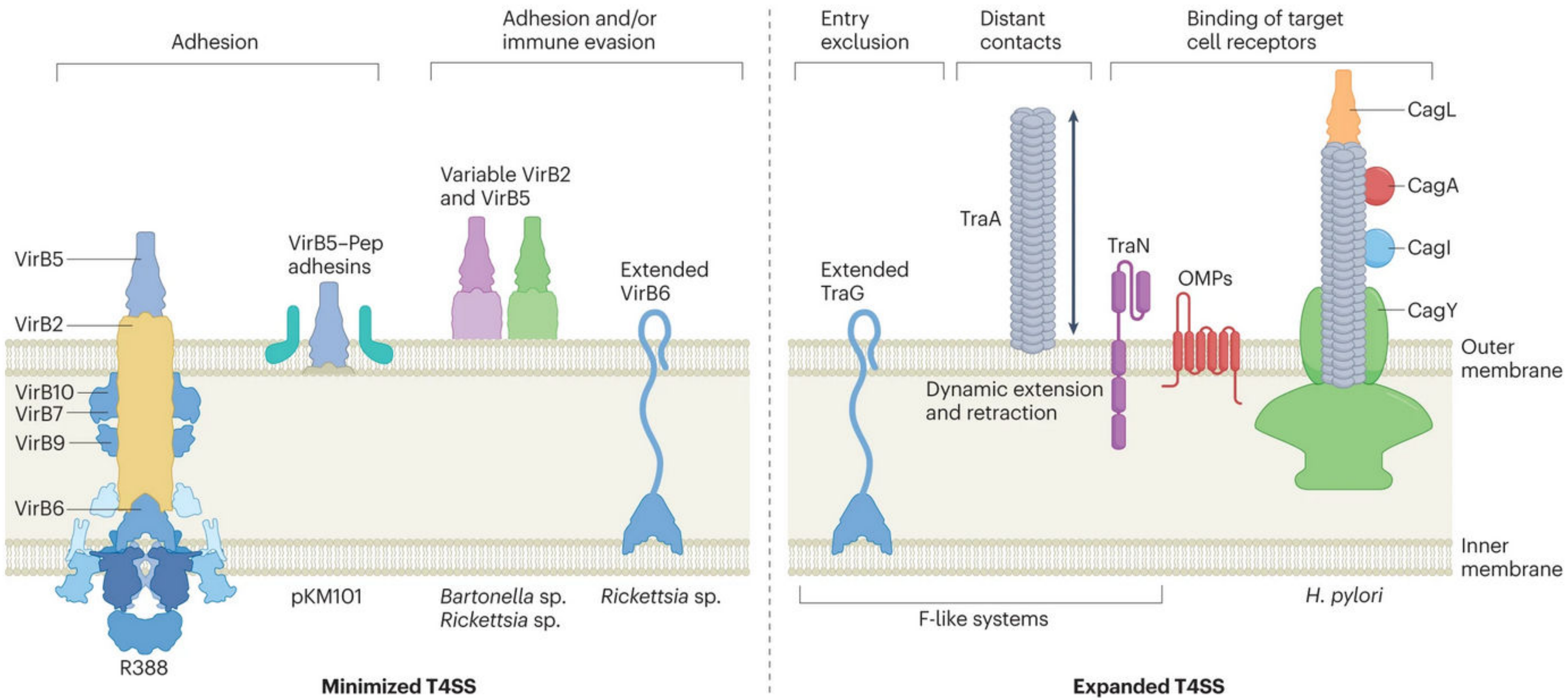
From: [Structural and functional diversity of type IV secretion systems](#)



a, Three expanded type IV secretion system (T4SS) outer membrane core complex (OMCC) structures are shown. The OMCCs of the F plasmid (Protein Data Bank (PDB) identifier (ID) 7OKN and PDB ID 7OKO), *Legionella pneumophila* (PDB ID 7MUS) and *Helicobacter pylori* (PDB ID 6X6S and 6X6J) are shown in surface representation and coloured in dark red, blue and green for the VirB7-like, VirB9-like and VirB10-like proteins, respectively, and in grey for other components. Notably, *L. pneumophila* and *H. pylori* OMCCs contain an outer membrane cap (OMC) and a periplasmic ring (PR). **b**, Cryo-electron tomography (cryo-ET) maps of the F plasmid with and without pilus. The maps (Electron Microscopy Data Bank (EMDB) entry 9344 and 9347) are coloured by sub-complexes (that is, green for the OMCC, red for the stalk, yellow for the arches, blue for the inner membrane complex (IMC) and grey for the pilus). The junction of the pilus and stalk is not well defined. **c**, Cryo-ET map of the *L. pneumophila* T4SS (EMDB entry 7611 and 7612) coloured as in part **b**, in front and slice view. **d**, Cryo-ET of the *H. pylori* T4SS (EMDB entry 0634 and 0635) coloured as in part **b**, in front and slice view.

Fig. 4: Examples of type IV secretion system subunit adaptations for functional diversification.

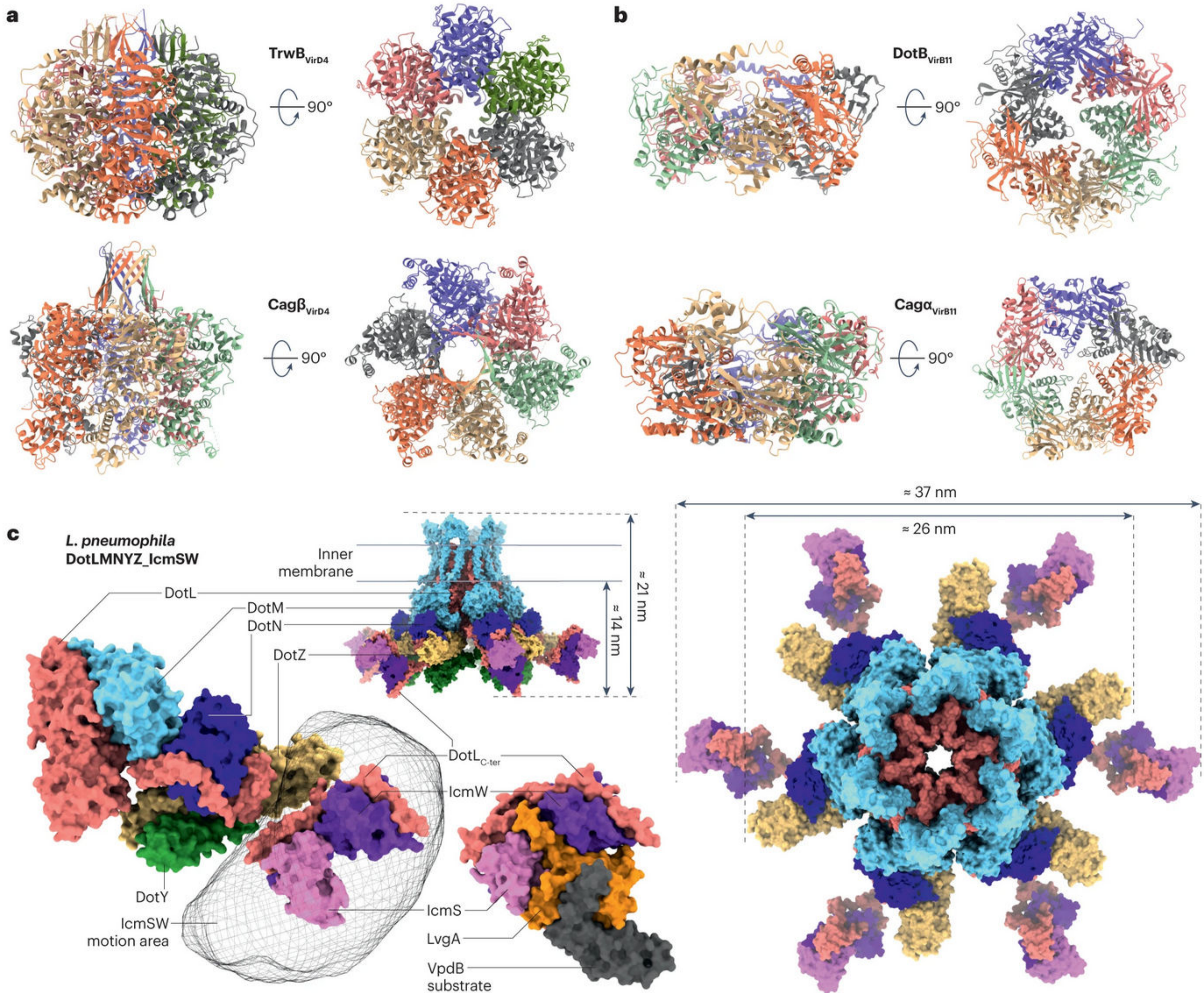
From: [Structural and functional diversity of type IV secretion systems](#)



The R388-encoded type IV secretion system (T4SS) is shown on the left for reference. VirB5 subunits are deployed for binding of target-cell receptors; these subunits can localize at the tips of conjugative pili or on the bacterial cell surface. Some bacteria encode several copies of VirB2 or VirB5 subunits whose variable sequences are postulated to bind different target-cell receptors or contribute to evasion of the host immune system. Extended VirB6 subunits carry large hydrophilic domains, several of which have been shown or are implicated in localizing at the cell surface to promote adhesion or immunomodulation, or blocking redundant plasmid transfer. F systems elaborate F pili that dynamically extend and retract to establish contacts with potential recipient cells at a distance. F systems also code for TraN subunits, whose extracellular domains interact with outer membrane proteins (OMPs) on recipient cells to promote F plasmid transfer and specify plasmid host range. Several T4SSs possess variant forms of the VirB7–VirB9–VirB10 core complex subunits, as exemplified for CagY in the *Helicobacter pylori* Cag system. The *H. pylori* system elaborates a conjugative pilus, which is decorated by other Cag subunits and the CagA secretion substrate. Various T4SSs functionally interact with other surface adhesins, such as pKM101-encoded Pep or *H. pylori* OMPs, to promote target-cell binding.

Fig. 5: Structure of VirD4-like and VirB11-like ATPases.

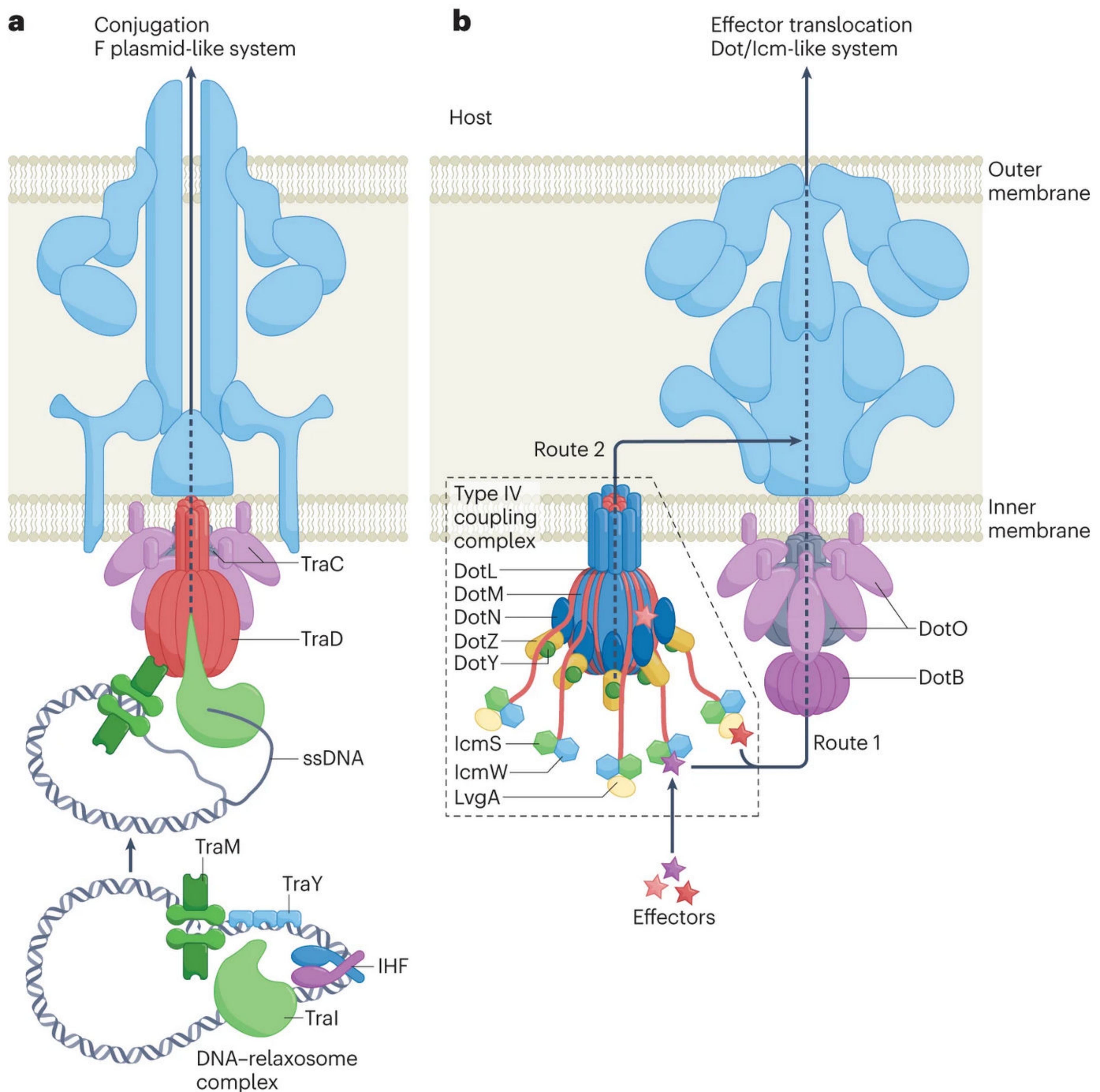
From: [Structural and functional diversity of type IV secretion systems](#)



a, Side view (left) and top view (right) of two VirD4-like ATPase structures: hexameric TrwB from R388 plasmid (Protein Data Bank (PDB) identifier (ID) 1GKI) and hexameric Cagβ from *Helicobacter pylori* (PDB ID 8DOL). Structures are shown in ribbon representation and coloured by monomer. **b**, Side view (left) and top view (right) of two VirB11-like ATPase structures; hexameric DotB from *Legionella pneumophila* (PDB ID 6GEF) and hexameric Cagα from *H. pylori* (PDB ID 1NLZ). Structures are shown in ribbon representation and coloured by monomer. **c**, Organization and structure of type IV coupling complex (T4CC) from *L. pneumophila*. The monomeric cryo-electron microscopy (cryo-EM) structure of the T4CC (PDB ID 6SZ9) is shown in surface representation and coloured by protein: VirD4-like DotL in red, DotM in cyan, DotN in blue, DotZ in yellow, DotY in green, IcmS in pink and IcmW in purple. The module made up of the extreme C terminus of (DotL_{C-ter}) and IcmSW is flexible, and its motion area is represented in mesh. Crystal structure of the DotL_{C-ter}-IcmSW module in the presence of LvgA adaptor (orange) and VpdB substrate (black) is shown (PDB ID 7BWK). On the right, a model of the hexameric T4CC structure is shown in top view using the same colour coding as in part **c**.

Fig. 6: Models for substrate recruitment and transport through the type IV secretion system.

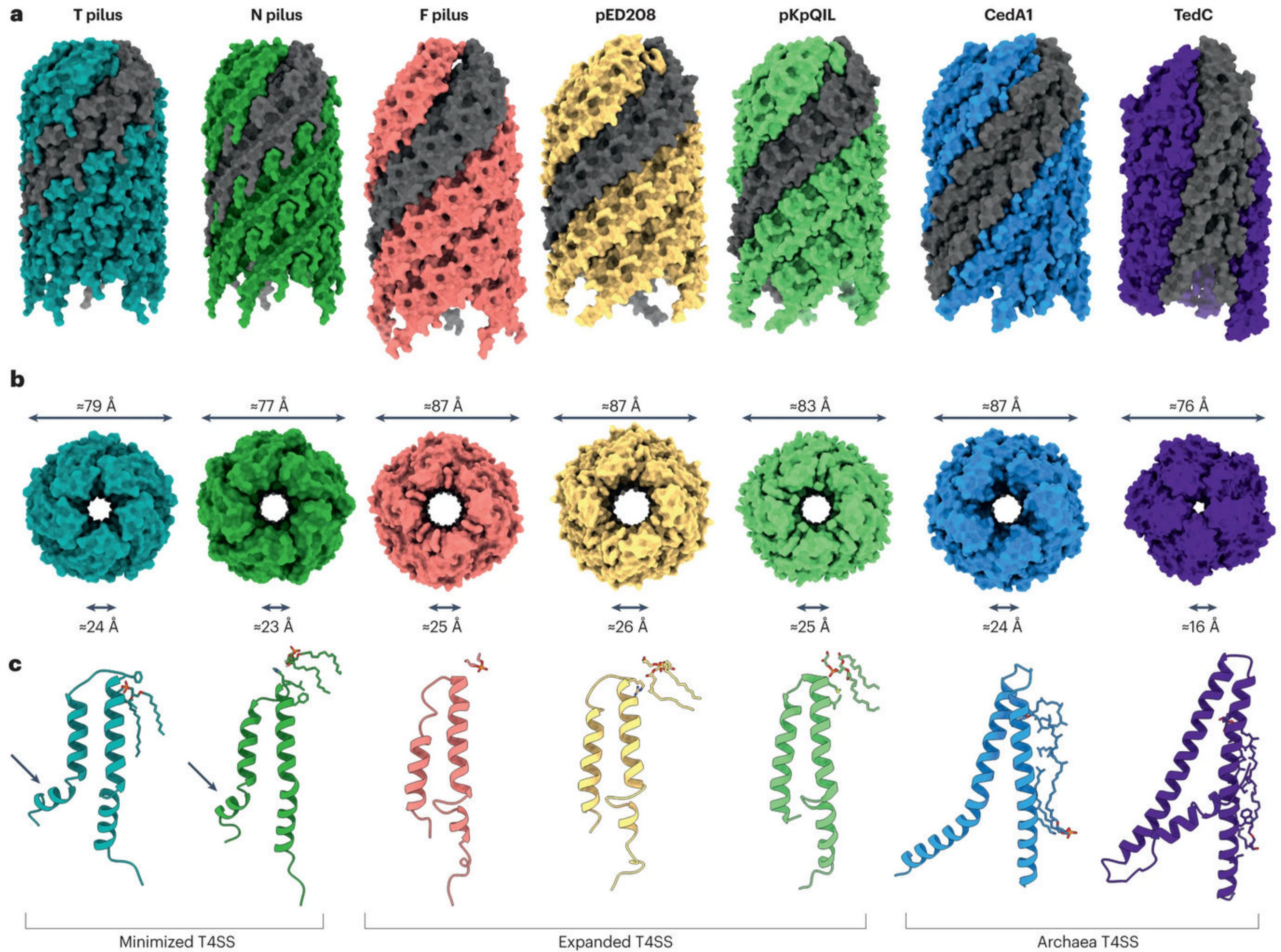
From: [Structural and functional diversity of type IV secretion systems](#)



a, Conjugative type IV secretion system (T4SS) recruitment and secretion mechanism model. Silhouette of F plasmid T4SS is shown in blue. First, the DNA is processed by a relaxosomal complex made of TraM (dark green), TraY (turquoise), IHF (purple and dark blue) and Tral (light green). The relaxosome is recruited by TraD_{VirD4} ATPase, which energizes the secretion of the Tral–single-stranded DNA (ssDNA) through the T4SS apparatus into the host. The relative position of TraD_{VirD4} and the global organization of the inner membrane complex during DNA secretion are unknown. **b**, T4SS effector recruitment and secretion mechanism model. Silhouette of *Legionella pneumophila* Dot/Icm T4SS is shown in blue. The type IV coupling complex (T4CC) acts as an effector recruitment platform and is schematically represented and positioned beside the complex formed by the hexameric dimers of DotO and DotB, although its precise localization is unknown. Effector proteins are captured by the T4CC at different binding sites and DotL_{VirD4} energizes substrate translocation via one of two possible routes across the inner membrane. Route 1: the T4CC feeds substrates into the DotO–DotB energy centre at the base of the T4SS channel for transit in one step across the entire cell envelope. Route 2: the T4CC feeds substrates into the lumen of the DotL hexamer for delivery across the inner membrane. In a second translocation step, substrates are recruited from the periplasm by the T4SS channel for passage to the cell surface and into target cells. Part **b** adapted with permission from ref. [28](#). Microbiology Society.

Fig. 7: Structure comparison between minimal, expanded and archaea pilus.

From: [Structural and functional diversity of type IV secretion systems](#)



a, Side view of all known pilus structures. Pilus structures (Protein Data Bank (PDB) identifier (ID) 8EXH *Agrobacterium tumefaciens* T pilus, PDB ID 8CW4 *Escherichia coli* N pilus, PDB ID 5LER *E. coli* F pilus, PDB ID 5LEG *Salmonella enterica* subsp. *enterica* serovar Typhimurium pED208, PDB ID 7JSV *Klebsiella pneumoniae* pKpQIL, PDB ID 8DFU *Aeropyrum pernix* CedA1 and PDB ID 8DFT *Pyrobaculum calidifontis* TedC) are in surface representation with one strand coloured in grey. **b**, Top view of pilus structures. Diameter and lumen sizes are indicated. **c**, For each pilus, one monomer of VirB2 with its lipid is shown in ribbon representation. The arrows in the minimized type IV secretion systems (T4SSs) highlight the presence of a 'kink', which is characteristic of this group.

Parts **a** and **b** adapted from ref. [108](#), CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

966 **Table of content:**

967 In this Review, Costa and colleagues summarise the current knowledge of type IV secretion system (T4SS)
968 functioning in Gram-negative bacteria, with a focus on their architectures and adaptations for specialised
969 functions. They also explore the biogenesis pathways and spatial localization of T4SSs.

970

971 **Glossary**

972

973 **Conjugative Pili:**

974 Helical hair-like appendages formed by protein-phospholipid complexes that assemble on the surface of bacteria
975 and can act as conduits for DNA transfer between donor and recipient bacteria.

976

977 **Biotic and abiotic adhesion:**

978 The attachment of microorganisms to living (biotic) or non-living (abiotic) surfaces, a process that typically
979 facilitates biofilm formation, niche establishment, or infection.

980

981 **Bacterial conjugation:**

982 Type of horizontal gene transfer in bacteria where genetic material, such as plasmids containing genes for
983 antibiotic resistance, is transferred from a donor bacterium to a recipient bacterium.

984

985 **Effector:**

986 Bacterial protein, often secreted through a dedicated secretion system, that interacts with and manipulates
987 cellular processes within a host organism, promoting bacterial survival, colonization, or infection.

988

989 **Cryo-electron microscopy (cryo-EM):**

990 Electron microscopy imaging technique that involves freezing samples in vitreous ice to preserve their native
991 state and is used to visualize the three-dimensional structure of biological molecules and complexes at near-
992 atomic resolution.

993

994 **Conjugative plasmid:**

995 A type of a bacterial plasmid that encodes a conjugative machinery, through which the plasmid and its cargoes
996 of antimicrobial resistance genes, virulence factors, or other fitness traits are delivered between bacterial cells.

997

998 **Polytopic topology:**

999 Protein structure that contains multiple transmembrane segments embedded in the cell membrane.
1000
1001 **Co-evolution of proteins:**
1002 The reciprocal influence and evolution patterns between two proteins that interact or are dependent on each
1003 other for function, and have evolved in a coordinated, non-random manner reflecting their mutual adaptation
1004 over time.
1005
1006 **Site-directed mutagenesis:**
1007 Molecular biology technique used to introduce specific nucleotide mutations into DNA sequences, with the
1008 purpose of studying their effects on protein structure and function.
1009
1010 **Cryo-electron tomography (cryo-ET):**
1011 Specialized variation of cryo-EM that enables the visualization of large cellular components or organelles within
1012 their cellular environment.
1013
1014 **Cryo-focused ion beam (cryo-FIB):**
1015 A technique used to prepare samples for cryo-electron microscopy by thinning frozen samples with a focused
1016 ion beam, leading to an improved signal-to-noise ratio and resolution in the imaging of biological samples.
1017
1018 **Toll-like receptor:**
1019 A family of pattern recognition receptors (PRR) in the immune system that specifically recognize conserved
1020 patterns in pathogens and triggers an immune response.
1021
1022 **Crosslinking:**
1023 Artificial formation of covalent bonds by a crosslinker between different molecules that interact or co-localize
1024 within a biological sample, with the common application of studying protein-protein or protein-ligand
1025 interactions.
1026
1027 **Rotational raise:**
1028 Angle at which adjacent rings stack in helical assemblies, influencing the overall helical symmetry and packing
1029 of the structure.
1030
1031 **Translocation signal:**
1032 Specific amino acid sequence that confers recognition of a protein as a substrate for a dedicated transport
1033 machinery for delivery to a specific cellular location, the extracellular milieu, or another bacterial or eukaryotic
1034 cell.
1035
1036 **Correlative light and electron microscopy (CLEM):**

1037 Imaging technique that combines fluorescence microscopy and electron microscopy to correlate high-resolution
1038 structural information with specific molecular or cellular labelling in the same sample.

1039

1040 **Nanobodies:**

1041 Single-domain antibody fragments derived from heavy-chain-only IgG antibodies that are naturally found in the
1042 Camelidae family, which includes camels, llamas, and alpacas.

1043

1044 **Adhesins:**

1045 Proteins found on the surface of cells that facilitate attachment to other biotic or abiotic surfaces.

1046

1047 **Biofilm:**

1048 An assemblage of bacteria on a biotic or abiotic surface, often with a defined architecture, that is embedded in
1049 an extracellular matrix typically composed of proteins, DNA, lipids and other biological molecules.

1050

1051 **Relaxosome:**

1052 A complex of proteins responsible for specific nicking of the double-stranded DNA, unwinding of DNA strands
1053 and delivering the single-stranded DNA transfer intermediate to the type IV secretion apparatus prior to
1054 conjugation.

1055