



HAL
open science

The type II secretion system – a dynamic fiber assembly nanomachine

Manuel Campos, David Cisneros, Mangayarkarasi Nivaskumar, Olivera Francetic

► To cite this version:

Manuel Campos, David Cisneros, Mangayarkarasi Nivaskumar, Olivera Francetic. The type II secretion system – a dynamic fiber assembly nanomachine. *Research in Microbiology*, 2013, 164 (6), pp.545-555. 10.1016/j.resmic.2013.03.013 . hal-02351800

HAL Id: hal-02351800

<https://hal.science/hal-02351800>

Submitted on 3 Jun 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The type II secretion system - a dynamic fiber assembly nanomachine

Manuel Campos^{a,c}, David A. Cisneros^b, Mangayarkarasi Nivaskumar^a and Olivera Francetic
Institut Pasteur, Molecular Genetics Unit, Department of Microbiology, 75015 Paris, France.
CNRS ERL3526, 75724 Paris, France.

^aUniversity Paris VII, 25 rue du Dr Roux, 75724 Paris CEDEX 15

^bPresent address: Research Institute of Molecular Pathology (IMP), Dr. Bohr-Gasse 7, A-1030
Vienna, Austria and Max F. Perutz Laboratories (MFPL), University of Vienna, Dr. Bohr-
Gasse 9, A-1030 Vienna, Austria

^cPresent address: Yale University School of Medicine, New Haven, CT, USA

Abstract (100 w)

Type II secretion systems (T2SSs) share common origins and structure with archaeal flagella (archaella) and pili, bacterial competence systems and type IV pili. All of these systems use a conserved ATP-powered machinery to assemble helical fibers that are anchored in the plasma membrane. The T2SSs assemble pseudopili, periplasmic filaments that promote extracellular secretion of folded periplasmic proteins. Comparative analysis of T2SSs and related fiber assembly nanomachines might provide important clues on their functional specificities and dynamics. This review focuses on recent developments in the study of pseudopilus structure and biogenesis, and discusses mechanistic models of pseudopilus function in protein secretion.

22
23 **Introduction**

24
25 The type 2 secretion systems (T2SSs) translocate folded proteins from the periplasm of Gram-
26 negative bacteria across the outer membrane *via* a large channel that remains closed in the
27 resting state (reviewed by P. Howard in this issue). Upon secretion, the exoprotein substrates
28 are released or exposed on the bacterial surface (Rondelet and Condemine, this issue) to
29 perform diverse functions in macromolecule degradation, adhesion, electron transport or
30 pathogenesis. The remarkable progress made in the study of T2SS structure and organization
31 during the past decade has been described in detail in several recent reviews (Douzi *et al.*,
32 2012, Korotkov *et al.*, 2012, McLaughlin *et al.*, 2012). This review focuses on the core
33 function of T2SS - the assembly of periplasmic filaments called the pseudopili, which play an
34 essential but poorly understood function in promoting the protein transport.

35 The T2SSs belong to an ancient and widespread superfamily of membrane nano-
36 machines that specialize in the assembly of fibers built with subunits localized in the inner
37 membrane. This highly successful and versatile molecular device is found in all prokaryotes,
38 suggesting its ancient evolutionary origin. In archaea these systems form the base of archaella
39 (formerly archaeal flagella) (Jarrel and Albers, 2012), type IV-like pili and bindosomes, the
40 carbohydrate "capture" systems. In bacteria, beside the natural competence systems and T2SSs,
41 they are also found in the form of type IV pili (T4P), long flexible fibers extending from the
42 bacterial surface. T4P are involved in bacterial motility over solid substrates called twitching
43 motility, and in other functions including adhesion, DNA uptake, aggregation, biofilm
44 formation, signaling and protein transport (Pelacic, 2008).

45 All these diverse machineries have a common basic function - the assembly of helical
46 fibers from the plasma membrane localized pilin subunits. The pilin transmembrane (TM)
47 segments are highly conserved and comprise at the N-terminus a positively charged signal

48 anchor followed by a Gly or Ala residue, the cleavage site for a specific processing enzyme, the
49 prepilin peptidase. The hydrophobic segment extends into a long α -helical stem that is shielded
50 on one side by a globular domain composed of 4 to 5 anti-parallel β -strands separated by
51 different conserved or variable loop regions. Globular pilin domains are exposed on the surface
52 of assembled fibers with their variable sequence providing the fibers with diverse surface and
53 binding properties.

54 The striking primary sequence conservation of TM α -helical regions is the signature
55 allowing unambiguous identification of fiber assembly systems in bacterial and archaeal
56 genomes (Albers and Pohlschroder, 2009). Such sequence conservation suggests that a strong
57 selective pressure shaped these segments, which play crucial roles in several steps of the fiber
58 biogenesis pathway (Fig. 1). Their hydrophobicity is essential for interactions with the signal
59 recognition particle (Fig. 1a) and for co-translational targeting to the Sec translocase that
60 promotes pilin insertion in the membrane in an N-in - C-out orientation (Fig. 1b) (Arts *et al.*,
61 2007b, Francetic *et al.*, 2007). The specific prepilin peptidase removes the positively charged
62 signal anchor after the conserved Gly residue (Fig. 1c), and, in T4a pili and T2SS transfers a
63 methyl group to the new N-terminal residue. In the following, still poorly understood steps of
64 fiber biogenesis, the TM segment might mediate interactions with the conserved fiber assembly
65 factors in the membrane, including the polytopic IM protein of the PulF/PilC family (Fig. 1d).
66 Finally, the TM segments make contacts with neighboring pilins during fiber assembly and
67 form the hydrophobic core of assembled filaments (Fig. 1e).

68

69 **T2SS and fiber assembly nanomachines**

70

71 In the T2SS, between 12 and 15 genes are required for protein secretion. Their
72 organization is similar in different bacterial groups (Douzi *et al.*, 2012). In most species these

73 genes form a single operon *gspCDEFGHIJKLMNO* (for general secretion pathway) (Fig. 2a),
74 with different additional genes required for the assembly and targeting of the secretin channel
75 GspD subunits. Each *gsp* gene is essential for protein secretion, with the exception of *gspN*,
76 which is also absent from many bacteria. While this secretion-null phenotype did not favor the
77 genetic analysis of T2SSs, biochemical dissection of the system led to the current model of
78 T2SS organization in the bacterial envelope. Furthermore, the three-dimensional structure of
79 most soluble domains of T2SS components has been determined (McLaughlin et al., 2012,
80 Korotkov et al., 2012).

81 The functional studies of the T2SS have benefited from the comparative analysis with
82 type IV pili (T4P), the most prominent and best-studied members of the helical fiber assembly
83 nanomachine family. These thin and flexible filaments are found on the surface of many Gram-
84 negative and some Gram-positive bacteria (Pelicic, 2008). In *Pseudomonas aeruginosa*, the
85 core genes essential for TP4 biogenesis are organized in an operon *pilABCD*, encoding the
86 major pilin subunit PilA, a hexameric ATPase PilB, a polytopic membrane protein PilC and the
87 prepilin peptidase PilD. This basic fiber assembly module is found in all related systems,
88 including the T2SS, suggesting that pilus assembly and protein secretion, as well as natural
89 competence, share a common molecular mechanism (Hobbs and Mattick, 1993).

90 Five proteins contain an N-terminal prepilin peptidase cleavage site - the highly
91 abundant (major) GspG and low-abundance (minor) pseudopilins GspH, I, J and K. The
92 presence of these components and their processing and N-methylation by the prepilin peptidase
93 GspO suggested that T2SSs assemble short periplasmic fibers (Strom and Lory, 1991, Pugsley,
94 1993). These initially hypothetical structures, the pseudopili, could be visualized upon
95 overexpression of the *pul* genes encoding the *Klebsiella oxytoca* T2SS (Sauvonnet et al.,
96 2000b). Pseudopilus surface assembly allowed the biochemical and structural characterization
97 of these fibers and provided an additional phenotype for genetic analysis of the T2SS. Fiber

98 assembly is not specific to the Pul system: at least two others, the chitinase-specific Gsp T2SS
99 from *Escherichia coli* (Vignon *et al.*, 2003) and the *P. aeruginosa* Xcp system (Durand *et al.*,
100 2003) also show this property. In contrast, the Hxc system in *P. aeruginosa* does not assemble
101 surface pili under these conditions and seems to belong to a distinct T2SS subclass based on
102 phylogenetic studies (Durand *et al.*, 2011). While protein secretion strictly requires the
103 presence of all T2SS components except GspB and GspN, pilus assembly, studied under
104 overexpression conditions, does not require minor pseudopilins GpsH, GspI, GspJ and GspK,
105 and the inner membrane component GspM (Fig. 2) (Durand *et al.*, 2005). GspI has been
106 implicated in pseudopilus assembly initiation since reduced amount of pili is found in *gspI*
107 (*xcpV*) mutants (Sauvonnet *et al.*, 2000b, Durand *et al.*, 2005). Similar to T4P in *Neisseria* spp.
108 (Wolfgang *et al.*, 2000, Carbonnelle *et al.*, 2006), the secretin pore in the outer membrane is
109 not required for fiber assembly but rather for the surface presentation of assembled fibers. In
110 the absence of *K. oxytoca* PulD, PulG pili are assembled in the periplasm (Vignon *et al.*, 2003,
111 Cisneros *et al.*, 2011).

112

113 **The major pseudopilins and T2SS pili**

114

115 Only major pseudopilins can form long surface polymers, while minor pseudopilins fail to form
116 such fibers even when overproduced (Durand *et al.*, 2005). Although surface pili are considered
117 as an overproduction artifact, they facilitated the pseudopilus analysis using biochemical
118 approaches, fluorescence and electron microscopy (EM). When observed by negative staining
119 EM, pseudopili are highly similar to T4a pili, thin (≈ 6 nm), flexible and with bundling
120 propensity, which might result from the EM sample preparation (Sauvonnet *et al.*, 2000a,
121 Durand *et al.*, 2003). EM analysis of PulG pili shows a helical subunit arrangement: each
122 subunit is displaced relative to its neighbor in the fiber by a vertical shift (axial rise) of 10.4 Å

123 and a rotational (twist) angle of 84.7° (Kohler *et al.*, 2004), values similar to those found in T4a
124 pili (Craig *et al.*, 2006). X-ray crystallography of major pseudopilins showed a typical pilin
125 fold, with a long alpha-helical stem and an antiparallel 3-strand beta-sheet forming the globular
126 domain (Kohler *et al.*, 2004, Korotkov *et al.*, 2009). Interestingly, the C-terminal pseudopilin
127 domain is folded around a Ca^{2+} ion, forming a stable and well-structured loop (Korotkov *et al.*,
128 2009), which is also apparent in the NMR structure of major pseudopilin XcpT^{GspG} from *P.*
129 *aeruginosa* (Alphonse *et al.*, 2010). In *V. cholerae* the Ca^{2+} coordinating residues are essential
130 for protein secretion (Korotkov *et al.*, 2009), apparently without affecting the monomer
131 stability. In the Pul T2SS, equivalent mutations reduce dramatically the levels of PulG,
132 suggesting the involvement of Ca^{2+} in pseudopilin folding and stability (Campos *et al.*, 2010).
133 Interestingly, this loop has its counterpart in the variable loop of major T4a pilins that is
134 stabilized by a disulfide bridge (Craig *et al.*, 2006).

135 For T4a pili, a combination of cryo-EM and X-ray crystallography led to the first low-
136 resolution models of these fibers (Craig *et al.*, 2003). The first PulG pilus models, generated by
137 a similar approach revealed the presence of deep groves on the fiber surface (Kohler *et al.*,
138 2004). A prominent left-handed groove had been taken as evidence that PulG pili were
139 organized as a left-handed helix (Kohler *et al.*, 2004). However, the missing TM segments and
140 the overall lack of detail precluded the use of this model for further structure-function analysis.
141 The helical parameters (i.e. axial rise and rotation) from this study were used as constraints to
142 build another series of PulG pilus models using a flexible *ab initio* approach (Campos *et al.*,
143 2011). The 1000 models generated independently converged into a cluster of 200 highly similar
144 right-handed helix models. The analysis of inter-protomer distances in this model cluster
145 showed that the vast majority showed multiple highly conserved contacts (Campos *et al.*,
146 2010). Each PulG protomer (P) within the fiber is involved in intricate contacts with protomers
147 $P \pm 1$, $P \pm 3$, $P \pm 4$ and possibly $P \pm 7$ via different interfaces (Fig. 3). Biochemical analysis was

148 used to test these contacts and validate the models, but also to probe the function of different
149 interfaces. Site-directed mutagenesis and functional studies showed that the most extensive and
150 important contacts involve the two closest protomers (P and $P \pm 1$). In the model of gonococcal
151 T4a pilus, the key role in pilus assembly was attributed to electrostatic interactions between the
152 absolutely conserved pilin residue E5 of protomer P and the N-terminal amine group of the
153 nearest protomer above ($P+1$) (Craig et al., 2006). In the PulG pilus, in addition to this probable
154 contact, which cannot be tested experimentally, two important salt bridge interactions have
155 been identified involving negatively charged residues of the periplasmic α -helical stem (E44
156 and D48) and positively charged residues (R87 and R88) in a loop region of the globular major
157 pilin domain (Fig. 3). Highly conserved among the major pseudopilin family, these residues
158 were essential for both T2SS functions: protein secretion and pseudopilus assembly (Campos et
159 al., 2010). In contrast, mutations disrupting other interfaces differentially affect the two
160 functions and appear to play different roles, currently under investigation (Nivaskumar,
161 Campos *et al*, unpublished data).

162 The inter-protomer distances at the level of the hydrophobic pseudopilin segment have
163 been tested by double cysteine cross-linking studies. As predicted by the pseudopilus structural
164 models, the shortest distance in assembled pili is found between residues 10 ($P+1$) and 16 (P).
165 Placing Cys residues at these positions leads to covalent cross-linking of pili resulting in
166 detergent-resistant PulG multimers. Interestingly, a lower degree of crosslinking is observed
167 when cysteine residues are placed in neighboring positions, 9 and 16 on one hand, and 11 and
168 16 on the other. These mutually exclusive interactions suggest an iris-like movement of pilin
169 monomers in assembled fibers (Campos et al., 2010). Similar structural heterogeneity is
170 observed in archaella (Yu et al., 2012) and archeal pili (Wang et al., 2008) suggesting that other
171 related fibers undergo similar conformational changes. External forces might promote these
172 changes during assembly/disassembly and might propagate along the fibers, providing the

173 structural basis for a potential signaling mechanism, one of the major functions observed in
174 T4P.

175

176 **Minor pseudopilins**

177

178 Four low-abundance (minor) pseudopilins are essential for protein secretion under
179 physiological expression conditions. In contrast, surface pili are produced, although less
180 efficiently, in their absence when the components of the system are overproduced, leading to
181 the idea that minor subunits provide determinants on the pseudopilus surface that are involved
182 in secretion-specific interactions. In the simplest model, the secreted substrates interact directly
183 with the minor pseudopilins. However, since complementation studies suggest that minor
184 pseudopilins are not substrate specificity determinants {Possot, 2000 #29}, interactions with
185 other T2SS factors and alternative functions such as the secretin channel gating, regulation of
186 fiber length were proposed (Forest, 2008). Although extensive efforts were made to detect
187 minor pseudopilins in surface fibers, there is still no biochemical or microscopy-based evidence
188 for their co-assembly into pili in T2SS (Vignon et al., 2003). Nevertheless, they might be
189 present in periplasmic pseudopili under physiological conditions, as suggested by studies of the
190 *Xanthomonas campestris* T2SS (Kuo et al., 2005, Hu et al., 2002). In T4P, the equivalent minor
191 pilins have been detected in pilus fractions and visualized by immuno-EM (Giltner et al., 2010,
192 Winther-Larsen et al., 2005).

193 The most compelling argument for minor pseudopilin co-assembly into fibers is
194 provided by the structural studies. The crystal structure of the complex formed by the
195 periplasmic domains of EpsI and EpsJ from *Vibrio cholerae* indicated a 1-nm shift between the
196 two proteins, identical to that in assembled pili (Yanez et al., 2008, Lam et al., 2009, Kohler et
197 al., 2004). Furthermore, structural analysis of the GspJ-GspI-GspK trimer from enterotoxigenic

198 *E. coli* showed that they formed a pilus-like complex (Korotkov and Hol, 2008) with a 1-nm
199 axial rise and a rotational angle of 100° between neighboring subunits. An alpha-helical
200 domain of GspK inserted in the pilin β -sheet fold caps this complex, such that additional
201 subunits cannot fit on top. Therefore, GspK is likely to be localized at the tip of a putative
202 pseudopilus. Consistent with this model, the absolutely conserved residue E5 that is predicted
203 to neutralize the N-terminal positive charge of the protomer above is lacking in GspK. The
204 fourth minor pseudopilin XcpU^{GspH} in *P. aeruginosa* interacts with the trimeric tip complex *in*
205 *vitro*, suggesting that minor pseudopilins form a quaternary complex (Douzi *et al.*, 2009).
206 Based on extensive analysis of binding affinities between their periplasmic domains, Douzi *et*
207 *al* proposed a pseudopilus assembly model in which GspI^{XcpV} plays a central nucleating role
208 and GspH^{XcpU} links this complex to the major pseudopilin (Douzi, 2009 #7). In addition,
209 structural modeling allowed fitting of EpsH subunit below the GspJ-GspI-GspK complex
210 (Yanez *et al.*, 2008), although direct binding of a GspG subunit also provided a satisfactory fit
211 (Korotkov and Hol, 2008).

212 Early *in vivo* studies had suggested the role of GspI in assembly initiation (Sauvonnnet *et*
213 *al.*, 2000b). Overproduction of PulK and its *Pseudomonas* homologue XcpX reduced pilus
214 assembly, while deletion of *xcpX* led to assembly of longer pili, leading to the idea that minor
215 pseudopilins control the fiber length (Vignon *et al.*, 2003, Durand *et al.*, 2005). Recent
216 systematic analysis of minor pseudopilin mutants by immunofluorescence microscopy revealed
217 that not only *pulK* mutants, but also *pulI* and *pulJ* mutants make longer and fewer pili (Cisneros
218 *et al.*, 2011). Removing all minor pseudopilins led to complete loss of piliation, suggesting a
219 role of the tip complex in pseudopilus assembly initiation. However, once initiated, pseudopilus
220 assembly seems to continue unabated under plate culture conditions, as a function of the
221 available pilin pool in the inner membrane. This observation further confirms the lack of active
222 retraction and length control, consistent with the absence of retraction ATPase in the T2SS.

223 The *gspH* mutants were identical to wild type cells with respect to pilus number, suggesting
224 that GspH does not play a role in initiation but rather at a later, elongation step, or in the
225 transition between initiation and elongation (Cisneros et al., 2012).

226 Mutants lacking all minor pseudopilins occasionally produced long pili, suggesting that
227 the assembly machinery spontaneously activates in rare cases and that minor pseudopilins play
228 a catalytic role. The minor pseudopilin pair GspJ-GspI is sufficient to restore a significant level
229 of piliation in these mutants, consistent with the central position of these proteins in the
230 quaternary pseudopilin complex (Cisneros et al., 2011, Douzi et al., 2009). *In vivo* they form a
231 staggered complex, as evidenced by close contacts between TM segment residues 10 (GspI)
232 and 16 (GspJ) shown by cysteine cross-linking (Cisneros et al., 2011). These contacts are
233 determined by a specific fit between the globular domains of these proteins, which form an
234 extensive interacting surface (Korotkov and Hol, 2008). Molecular dynamics simulations,
235 mutational and cross-linking studies provide evidence for self-assembly of tip pseudopilins: the
236 staggered GspJ-GspI complex bends in the inner membrane to facilitate the binding of GspK
237 alpha domain to its upper surface; this in turn facilitates the extraction of PulK, exerting a
238 pulling force on the membrane. The strong correlation between GspJ-GspI-GspK binding and
239 their ability to promote assembly initiation, suggests that these proteins transduce a signal to
240 activate the assembly ATPase in the cytoplasm. How this is signal transduced and which
241 protein-protein interactions participate in activation, are some of the major unresolved
242 questions. Biochemical and structural studies from the Sandkvist and Hol labs in particular
243 provide some clues about the possible molecular mechanism of these events, discussed below.

244

245 **The pseudopilus assembly machinery**

246

247 The energy for the pseudopilus and T4P assembly is generated by conformational changes of

248 the assembly ATPase of the GspE/PilB family. These hexameric ATPases form a ring-like
249 structure with a central opening, leading initially to the idea that pilins are translocated to the
250 periplasm *via* this channel (Camberg and Sandkvist, 2005). X-ray structures of EpsE from *V.*
251 *cholerae* (Robien *et al.*, 2003) and XpsE from *X. campestris* (Chen *et al.*, 2005) show a bilobed
252 monomer structure, with the ATP binding site at the hinge region between the N-terminal and
253 C-terminal domains. In the recent EpsE hexamer model, the NTD and CTD of neighboring
254 subunits share extensive contacts (Patrick *et al.*, 2011). In this arrangement, which was tested
255 by site-directed mutagenesis, several arginine residues around the active site belonging to both
256 protomers seem to be required for ATP hydrolysis. Variants with alanine substitutions at these
257 positions are specifically defective in ATP hydrolysis but not in ATP binding or GspE
258 oligomerization. The strongest defect was associated with mutation R225A, in close proximity
259 to the bound nucleotide both in *V. cholerae* EpsE and in *X. campestris* ATPase XspE,
260 suggesting a direct role in ATP hydrolysis (Shiue *et al.*, 2007).

261 In the hexamer, two neighboring subunits contribute to ATP binding and hydrolysis,
262 resulting in three ATPase active sites (Patrick *et al.*, 2011). Structural insight into their
263 conformations comes from the studies of the closely related retraction ATPase PilT (Satyshur
264 *et al.*, 2007). Each PilT monomer pair shows a different active site conformation,
265 corresponding to the ATP-bound, hydrolysis and release state (Misic *et al.*, 2010). Extensive
266 rotation of the N-terminal domain around the active site hinge is involved in the transition
267 between the extreme states. Although these conformational states might appear stochastically, a
268 sequential model would be consistent with the biological data, suggesting that the addition of
269 one pseudopilin at a time at the level of the inner membrane drives both fiber polymerization
270 and protein secretion (Campos *et al.*, 2010). Interestingly, the subunit arrangement in the
271 initiation complex GspJ-GspI-GspK (Korotkov and Hol, 2008) mirrors the three-state ATPase
272 model and might use the self-assembly energy or fit or induce the three-state ATPase motor

273 (Cisneros et al., 2011), thus contradicting the three-start helix assembly model proposed for
274 T4P (Craig et al., 2006).

275 How are these conformational changes transduced to promote pseudopilus assembly?
276 One candidate for this role is GspF, the core fiber assembly component, whose interaction with
277 GspE has been demonstrated by two-hybrid and biochemical approaches (Py *et al.*, 2001).
278 GspF has three TM segments and two large cytoplasmic domains structured as 6 α -helix
279 bundles (Abendroth *et al.*, 2009). Little is known about the overall organization and
280 oligomerization state of GspF. Its homologue in T4P, PilG, forms dimers and tetramers whose
281 cryo-EM structure looks like a conical ring (Collins *et al.*, 2007). Interactions of GspF of *P.*
282 *aeruginosa* with GspL and GspE homologues are suggested by their mutual stabilization (Arts
283 *et al.*, 2007a) and by the yeast two-hybrid studies (Py et al., 2001). Details of these interactions
284 and their functional significance are still a major black box.

285 The N-terminal domain of GspE forms a stable complex with the cytoplasmic domain
286 of GspL, characterized by X-ray crystallography (Abendroth *et al.*, 2005). GspL is the
287 membrane anchor for the ATPase and favors its oligomerization. It also regulates the
288 interactions with membrane acidic phospholipids, phosphatidyl glycerol and cardiolipin, which
289 enhance GspE ATPase activity dramatically (Camberg et al., 2007).

290 Biochemical, two-hybrid and live imaging approaches provide ample evidence for
291 interactions between GspL and GspM (Py et al., 2001, Possot et al., 2000, Buddelmeijer et al.,
292 2006). In addition to its stabilizing effect on GspL, the C-terminal domain of GspM plays a
293 specific role in secretion (Camberg et al., 2007). Recent studies on *V. cholerae* T2SS show that
294 EpsL and EpsG can be cross-linked *in vivo* (Gray et al., 2011), suggesting that EpsL transmits
295 the ATP-ase driven conformational changes to the major pseudopilin to promote fiber
296 assembly. The GspL-GspG interacting surface requires further investigation, since *epsG*
297 mutants defective in interaction with EpsL map at residues that form an intra-molecular

298 hydrogen bond and affect the stability of EpsG monomer. Other T2SS components might
299 interact with the major pseudopilin. In *N. meningitidis*, the major pilin subunit PilE interacts
300 with PilG (a GspF homologue) and PilO, which is homologous to GspM (Georgiadou et al.,
301 2012).

302

303 **Pseudopilus assembly and protein secretion**

304

305 T2SSs and T4P assembly systems are remarkably similar. Recent structural studies revealed
306 that all T2SS components have their equivalents in T4aPS (Ayers et al., 2010). The minor
307 pseudopilins, which are specifically required for protein secretion, have their counterparts in
308 minor T4 pilins. Another "secretion specific" component GspM has a structural equivalent in
309 the T4P PilO protein (Ayers et al., 2009).

310 Some components, like the prepilin peptidase, are functionally interchangeable between
311 the T4P and T2SSs. The Pul T2SS can assemble the major T4 pilin PpdD encoded by the
312 chromosome of *E. coli* and many other enterobacteria (Sauvonnet et al., 2000b, Kohler et al.,
313 2004). Furthermore, the *E. coli* minor pilins can promote the initiation of PpdD assembly in a
314 heterologous system, catalyzed by the T2SS assembly machinery (Cisneros et al., 2012),
315 suggesting a role of minor T4 pilins in pilus assembly initiation. Minor T4 pilins are required
316 for pilus assembly in the presence of retraction ATPase PilT. Mutants of *pilT* are piliated, due
317 to the spontaneous assembly initiation events, the high concentration of pilins in the membrane
318 and their extremely high stability. Indeed, unlike the T2SS pseudopili, which are unstable and
319 short under physiological conditions, T4 pili are so stable that an energy-dependent process is
320 required to undo the fibers and reset the system. While flexible modeling predicts a similar
321 electrostatic and hydrophobic interaction network in pseudopili and T4P, the covalent disulfide

322 bonds stabilizing the T4 pilin monomers might be responsible for this remarkable fiber stability
323 (Campos et al., 2011, Li et al., 2012).

324 Interestingly, the minor T4 pilins of *E. coli* can promote the assembly of PulG, albeit
325 less efficiently than the cognate minor pseudopilins (Cisneros et al., 2012). The absence of
326 homology and therefore of structural complementarity between the globular domains of T4
327 pilins and the major T2SS pseudopilin (and *vice versa*) suggests that their role in assembly
328 initiation does not rely on complex formation between these proteins. The only similarities
329 between T4 pilins and T2SS pseudopilins are found in their hydrophobic segments, suggesting
330 a role in crucial interactions with the assembly machinery. According to the activation model,
331 the minor T4 pilin or minor pseudopilin complex formation acts upon the assembly machinery
332 to start the first cycle of ATP binding and hydrolysis. However, pseudopilus assembly is more
333 efficient in the presence of cognate minor pseudopilins, suggesting that a scaffolding
334 mechanism (*via* direct binding of pseudopilin globular domains) contributes to assembly
335 initiation (Cisneros et al., 2012, Cisneros et al., 2011, Burrows, 2012). In support of this model,
336 full activation of pseudopilus assembly that leads to protein secretion is achieved in the
337 presence of cognate pseudopilins in the T2SS.

338

339 **Models of protein secretion**

340

341 How is the energy of pseudopilus assembly transduced to promote protein secretion?
342 Based on the similarities of T2SS and T4P, at least two models of protein secretion mechanism
343 have been proposed (Nunn, 1999) known as "the piston" and the "Archimedes' screw" models.
344 The idea of the piston was based on the dynamics of T4a pili, where cycles of pseudopilus
345 extension and retraction push the substrate through the secretin channel. According to this
346 model, pseudopilus assembly provides the driving force for secretion, acting as a piston, while

347 minor pseudopilins determine the binding specificity (Shevchik et al., 1997, Nunn, 1999,
348 Vignon et al., 2003). Major support for the piston model comes from recent findings that the
349 minor pseudopilin complex XcpU-V-W-X of *P. aeruginosa* binds *in vitro* to the specific
350 substrate of the Xcp system, LasB, but not to the substrate of the second Hxc system present in
351 the same species (Douzi *et al.*, 2011).

352 The major problem with this model is that in T2SS there is no evidence of fiber
353 retraction. Certain T4P assembly systems also secrete periplasmic proteins. One example is the
354 toxin co-regulated pilus (TCP) of *V. cholerae*, which secretes a soluble colonization factor
355 TcpF (Kirn *et al.*, 2003). Interestingly, the TCP system lacks the retraction ATPase, again
356 suggesting that pilus retraction is not required for protein secretion. A unidirectional assembly
357 might be required to ensure the vectorial transfer of proteins from the inner membrane towards
358 the exterior. The lack of retraction ATPase in T2SS led to the proposal that pseudopilus
359 "collapses" or is degraded, and that GspK might catalyze the degradation of major pseudopilin
360 (Durand et al., 2005). Indeed, pseudopilins might be more prone to degradation than T4 pilins.
361 Their Ca²⁺-coordinating C-terminal loop, structurally equivalent to the disulfide loop in T4
362 pilins, might provide a basis for the control of subunit structural integrity (Korotkov et al.,
363 2009).

364 In T4P and all helical fibers of this superfamily, subunits are shifted by a 1-nm
365 translation and variable rotation angles, with a symmetry that does not match that of the GspE
366 ATPase. This implies that, during assembly, the fibers rotate relative to the assembly ATPase
367 during addition of each new subunit (Mattick, 2002). The T2SS might be regarded as a
368 modified reverse ATP synthase that uses the energy release upon ATP hydrolysis for rotation
369 instead of generating ATP using the proton motive force (Streif *et al.*, 2008). The Archimedes'
370 screw model postulates exoprotein binding to the pseudopilus at the base, suggesting that major
371 pseudopilins provide the binding surface (Fig. 4). Addition of pseudopilin subunits would be

372 coupled to the rotary motion of the fiber and to a net upward transfer of subunits and bound
373 exoproteins from the pseudopilus base into the secretin channel. So far, there is less
374 biochemical support for this model than for the piston model. However, the exoprotein PulA of
375 the *K. oxytoca* T2SS interacts with the PulG pili (Kohler et al., 2004) (Francetic et al,
376 submitted). In addition, major pseudopilin mutations lead to a change in substrate specificity in
377 Xcp and Hxc, the two T2SS of *P. aeruginosa* (Durand et al., 2011).

378 Evidence for rotation during or upon assembly of helical fibers comes from archaella,
379 which rotate to promote swimming (Ghosh and Albers, 2011, Herzog and Wirth, 2012); S.V.
380 Albers, personal communication). While bacterial flagella require proton motive force for
381 rotation, archaella use the energy of ATP hydrolysis and do not seem to require motor proteins
382 other than the assembly machinery itself (Streif et al., 2008). Given their conservation, it is
383 plausible that all helical fiber assembly nanomachines of this superfamily display the same
384 dynamics. While rotation of rigid archaella allows them to "carry the weight" of the archaeal
385 cell and support swimming, rotation of flexible pseudopili or T4P might not be easily observed.
386 The ATPase GspE in T2SS is anchored to the membrane *via* GspL, but GspF might be free to
387 rotate in the membrane. Components of the fiber assembly platform anchored in the cell wall
388 might play a role of the stator, and the link with GspC and GspD might be important in this
389 regard (Korotkov *et al.*, 2011).

390 Clearly, the interactions between the membrane components of fiber assembly
391 nanomachines and fiber dynamics are a vast unexplored area that awaits further research.
392 Comparative analysis of T2SSs, T4P, archaeal pili and archaella should provide important
393 clues on the common mechanisms and significant differences that underlie the specialized
394 functions of these remarkable systems.

395

396 **Acknowledgments**

397 The study of protein secretion in our team has been funded by the Institut Pasteur grant
 398 PTR339. MC was a scholar of the French Ministry of Science and Education, MN is the
 399 recipient of Pasteur-Paris University fellowship and DC was funded by EMBO long term and
 400 Roux fellowships. We are grateful to our collaborators Michaël Nilges, Guillaume Bouvier,
 401 Peter J. Bond, Gérard Péhau-Arnaudet, Nadia Izadi-Pruneyre, Daniel Ladant and Gouzel
 402 Karimova for their contributions to the pseudopilus project. We are grateful to all members of
 403 the Molecular Genetics Unit for many stimulating discussions. We thank Tony Pugsley for
 404 generous support and for the critical reading of the manuscript.

405
 406 **References**
 407

- 408 Abendroth, J., D. Mitchell, K. Korotkov, T. Johnson, A. Kreger, M. Sankvist and W. Hol,
 409 2009. The three-dimensional structure of the cytoplasmic domains of EpsF from the
 410 type 2 secretion system of *Vibrio cholerae*. *J Struct Biol* 166, 303-315.
- 411 Abendroth, J., P. Murphy, M. Sandkvist, M. Bagdasarian and W. G. Hol, 2005. The X-ray
 412 structure of the type II secretion system complex formed by the N-terminal domain of
 413 EpsE and the cytoplasmic domain of EpsL of *Vibrio cholerae*. *J Mol Biol* 348, 845-855.
- 414 Albers, S. V. and M. Pohlschroder, 2009. Diversity of archaeal type IV pilin-like structures.
 415 *Extremophiles* 13, 403-410.
- 416 Alphonse, S., E. Durand, B. Douzi, B. Waegele, H. Darbon, A. Filloux, R. Voulhoux and C.
 417 Bernard, 2010. Structure of the *Pseudomonas aeruginosa* XcpT pseudopilin, a major
 418 component of the type II secretion system. *J. Struct. Biol.* 169, 75-80.
- 419 Arts, J., A. de Groot, G. Ball, E. Durand, M. El Khattabi, A. Filloux, J. Tommassen and M.
 420 Koster, 2007a. Interaction domains in the *Pseudomonas aeruginosa* type II secretory
 421 apparatus component XcpS (GspF). *Microbiol* 153, 1582-1592.
- 422 Arts, J., R. van Boxtel, A. Filloux, J. Tommassen and M. Koster, 2007b. Export of the
 423 pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the
 424 signal recognition particle-Sec pathway. *J Bacteriol.* 189, 2069-2076.
- 425 Ayers, M., P. L. Howell and L. L. Burrows, 2010. Architecture of the type II secretion and type
 426 IV pilus machineries. *Future Microbiol* 5, 1203-1218.
- 427 Ayers, M., L. Sampaleanu, S. Tammam, J. Koo, H. Harvey, P. Howell and L. Burrows, 2009.
 428 PilM/N/O/P proteins form an inner membrane complex that affects the stability of the
 429 *Pseudomonas aeruginosa* type IV pilus secretin. *J Mol. Biol.* 394, 128-142.
- 430 Buddelmeijer, N., O. Francetic and A. P. Pugsley, 2006. Green fluorescent chimeras indicate
 431 nonpolar localization of pullulanase secretion components Pull and PulM. *J.*
 432 *Bacteriol.* 188, 2928-2935.
- 433 Burrows, L. L., 2012. Prime time for minor subunits in type II secretion and type IV pilus
 434 systems. *Mol Microbiol* 86.
- 435 Camberg, J. L., T. L. Johnson, M. Patrick, J. Abendroth, W. G. Hol and M. Sandkvist, 2007.
 436 Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids.
 437 *EMBO J* 26, 19-27.

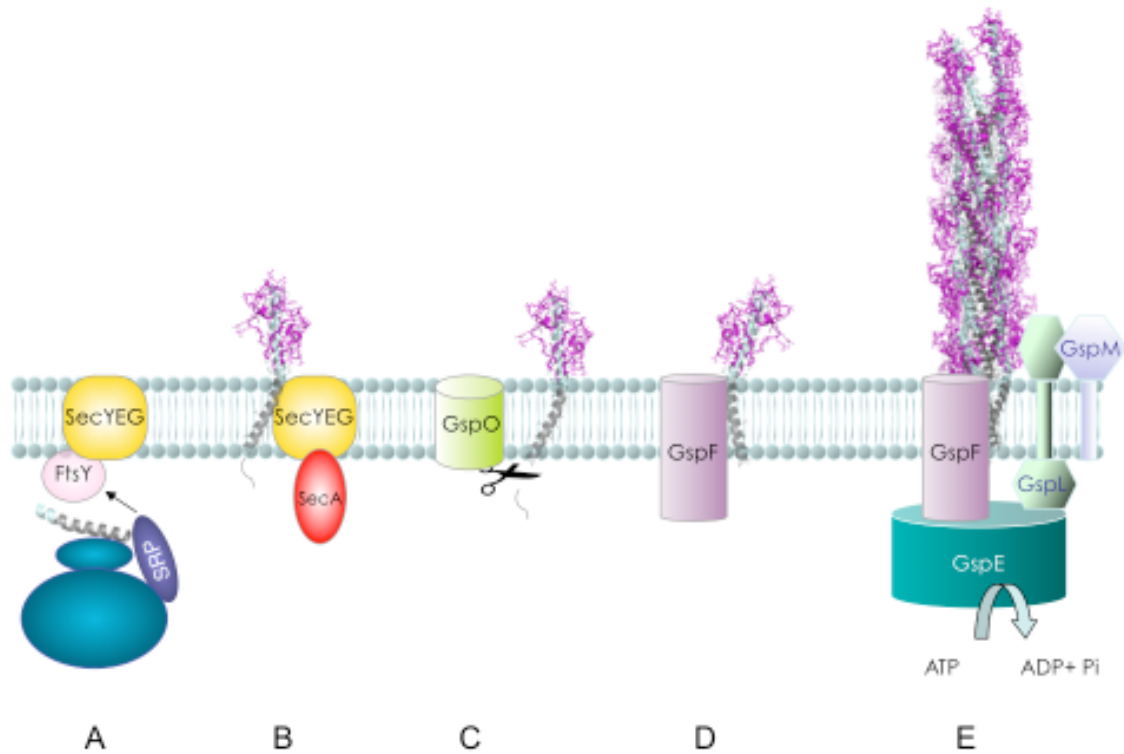
- 438 Camberg, J. L. and M. Sandkvist, 2005. Molecular analysis of the *Vibrio cholerae* type II
439 secretion ATPase EpsE. *J Bacteriol* 187, 249-256.
- 440 Campos, M., O. Francetic and M. Nilges, 2011. Modeling pilus structures from sparse data. *J*
441 *J Struct Biol.* 173, 436-444.
- 442 Campos, M., M. Nilges, D. A. Cisneros and O. Francetic, 2010. Detailed structural and
443 assembly model of the type II secretion pilus from sparse data. *Proc Natl Acad Sci*
444 *USA* 107, 13081-13086.
- 445 Carbonnelle, E., S. Helaine, X. Nassif and V. Pelicic, 2006. A systematic genetic analysis in
446 *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality,
447 stabilization and export of type IV pili. *Mol Microbiol* 61, 1510-1522.
- 448 Chen, Y., S. J. Shiue, C. W. Huang, J. L. Chang, Y. L. Chien, N. T. Hu and N. L. Chan, 2005.
449 Structure and function of the XpsE N-terminal domain, an essential component of the
450 *Xanthomonas campestris* type II secretion system. *J Biol Chem* 280, 42356-42363.
- 451 Cisneros, D., G. Péhau-Arnaudet and O. Francetic, 2012. Heterologous assembly of type IV
452 pili by a type II secretion system reveals the role of minor pilins in assembly
453 initiation. *Mol Microbiol* 86.
- 454 Cisneros, D. A., P. J. Bond, A. P. Pugsley, M. Campos and O. Francetic, 2011. Minor
455 pseudopilin self-assembly primes type II secretion pseudopilus elongation.
456 *EMBO J* 31, 1041-1053.
- 457 Collins, R. F., M. Saleem and J. P. Derrick, 2007. Purification and three-dimensional electron
458 microscopy structure of the *Neisseria meningitidis* Type IV pilus biogenesis protein
459 PilG. *J Bacteriol* 189, 6389-6396.
- 460 Craig, L., R. K. Taylor, M. E. Pique, B. D. Adair, A. S. Arvai, M. Singh, S. J. Lloyd, D. S.
461 Shin, E. D. Getzoff, M. Yeager, F. K.T. and J. A. Tainer, 2003. Type IV pilin structure
462 and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and
463 *Pseudomonas aeruginosa* PAK pilin. *Molec Cell* 11, 1139-1150.
- 464 Craig, L., N. Volkmann, A. S. Arvai, M. E. Pique, M. Yeager, E. H. Egelman and J. A. Tainer,
465 2006. Type IV pilus structure by cryo-electron microscopy and crystallography:
466 implications for pilus assembly and functions. *Mol Cell* 23, 651-662.
- 467 Douzi, B., G. Ball, C. Cambillau, M. Tegoni and R. Voulhoux, 2011. Deciphering the Xcp
468 *Pseudomonas aeruginosa* type II secretion machinery through multiple interactions
469 with substrates. *J Bio Chem* 286, 40792-40801.
- 470 Douzi, B., E. Durand, C. Bernard, S. Alphonse, C. Cambillau, A. Filloux, M. Tegoni and R.
471 Voulhoux, 2009. The XcpV/GspI pseudopilin has a central role in the assembly of a
472 quaternary complex within the T2SS pseudopilus. *J Biol Chem* 284, 34580-34589.
- 473 Douzi, B., A. Filloux and R. Voulhoux, 2012. On the path to uncover the bacterial type II
474 secretion system. *Phil Trans R Soc B* 367, 1059-1072.
- 475 Durand, E., S. Alphonse, C. Brochier-Armanet, G. Ball, B. Douzi, A. Filloux, C. Bernard and
476 R. Voulhoux, 2011. The assembly mode of the pseudopilus. A hallmark to distinguish a
477 novel secretion system subtype. *J. Biol. Chem.* 286, 24407-24416.
- 478 Durand, E., A. Bernadac, G. Ball, A. Lazdunski, J. N. Sturgis and A. Filloux, 2003. Type II
479 protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar and
480 adhesive structure. *J Bacteriol* 185, 2749-2758.
- 481 Durand, E., G. Michel, R. Voulhoux, J. Kurner, A. Bernadac and A. Filloux, 2005. XcpX
482 controls biogenesis of the *Pseudomonas aeruginosa* XcpT-containing pseudopilus. *The*
483 *J Biol Chem* 280, 31378-31389.
- 484 Forest, K. T., 2008. The type II secretion arrowhead: the structure of GspI-GspJ-GspK. *Nat*
485 *Struct Mol Biol* 15, 428-430.

- 486 Francetic, O., N. Buddelmeijer, S. Lewenza, C. A. Kumamoto and A. P. Pugsley, 2007. Signal
487 recognition particle-dependent inner membrane targeting of the PulG pseudopilin
488 component of a type II secretion system. *J Bacteriol* 189, 1783-1793.
- 489 Georgiadou, M., M. Castagnini, G. Karimova, D. Ladant and V. Pelicic, 2012. Large-scale
490 study of the interactions between proteins involved in type IV pilus biology in
491 *Neisseria meningitidis*: characterization of a sub-complex involved in pilus assembly.
492 *Mol Microbiol* 84, 857-873.
- 493 Ghosh, A. and S. V. Albers, 2011. Assembly and function of the archaeal flagellum. *Biochem*
494 *Soc Trans* 39, 64-69.
- 495 Giltner, C. L., M. Habash and L. L. Burrows, 2010. *Pseudomonas aeruginosa* minor pilins are
496 incorporated into type IV pili. *J Mol Biol* 398, 444-461.
- 497 Gray, M. D., M. Bagdasarian, W. G. Hol and M. Sandkvist, 2011. In vivo cross-linking of
498 EpsG to EpsL suggests a role for EpsL as an ATPase-pseudopilin coupling protein in
499 the Type II secretion system of *Vibrio cholerae*. *Mol Microbiol* 79, 786-798.
- 500 Herzog, B. and R. Wirth, 2012. Swimming behaviour of selected species of archaea. *Appl*
501 *Environ Microbiol.* 78, 1670-1674.
- 502 Hobbs, M. and J. S. Mattick, 1993. Common components in the assembly of type 4 fimbriae,
503 DNA transfer systems, filamentous phage and protein-secretion apparatus: a general
504 system for the formation of surface-associated protein complexes. *Mol. Microbiol.* 10,
505 233-243.
- 506 Hu, N. T., W. M. Leu, M. S. Lee, A. Chen, S. C. Chen, Y. L. Song and L. Y. Chen, 2002.
507 XpsG, the major pseudopilin in *Xanthomonas campestris* pv. *campestris*, forms a pilus-
508 like structure between cytoplasmic and outer membranes. *Biochem. J* 365, 205-211.
- 509 Jarrel, K. F. and S. V. Albers, 2012. The archeallum: an old motility structure with a new name.
510 *Trends Microbiol* 20, 307-312.
- 511 Kirn, T. J., N. Bose and R. K. Taylor, 2003. Secretion of a soluble colonization factor by the
512 TCP type 4 pilus biogenesis pathway in *Vibrio cholerae*. *Mol. Microbiol.* 49, 81-92.
- 513 Kohler, R., K. Schafer, S. Muller, G. Vignon, K. Diederichs, A. Philippsen, P. Ringler, A. P.
514 Pugsley, A. Engel and W. Welte, 2004. Structure and assembly of the pseudopilin PulG.
515 *Mol Microbiol* 54, 647-664.
- 516 Korotkov, K., T. L. Johnson, M. Jobling, J. Pruneda, E. Pardon, A. Héroux, S. Turley, J.
517 Steayert, R. Holmes, S. M and H. WG, 2011. Structural and functional studies on the
518 interactions of GspC and GspD in type II secretion system. *PLOS Pathog.*
- 519 Korotkov, K., M. Sandkvist and W. Hol, 2012. The type II secretion system: biogenesis,
520 molecular architecture and mechanism. *Nat Rev Microbiol* 10, 336-351.
- 521 Korotkov, K. V., M. Gray, A. Kreger, S. Turley, M. Sandkvist and W. Hol, 2009. Calcium is
522 essential for the major pseudopilin in the type 2 secretion system. *J Biol Chem* 284,
523 25466-25470.
- 524 Korotkov, K. V. and W. G. Hol, 2008. Structure of the GspK-GspI-GspJ complex from the
525 enterotoxigenic *Escherichia coli* type 2 secretion system. *Nat Struct Mol Biol* 15, 462-
526 468.
- 527 Kuo, W. W., H. W. Kuo, C. C. Cheng, H. L. Lai and L. Y. Chen, 2005. Roles of the minor
528 pseudopilins, XpsH, XpsI and XpsJ, in the formation of XpsG-containing pseudopilus
529 in *Xanthomonas campestris* pv. *campestris*. *J Biomed Sci* 12, 587-599.
- 530 Lam, A. Y., E. Pardon, K. V. Korotkov, W. G. Hol and J. Steyaert, 2009. Nanobody-aided
531 structure determination of the EpsI:EpsJ pseudopilin heterodimer from *Vibrio*
532 *vulnificus*. *J Struct Biol* 166, 8-15.
- 533 Li, J., E. Egelman and L. Craig, 2012. Structure of the *Vibrio cholerae* type IV b pilus and
534 stability comparison with *Neisseria gonorrhoeae* type IV a pilus. *J Mol Biol* 418, 47-64.
- 535 Mattick, J. S., 2002. Type IV pili and twitching motility. *Annu Rev Microbiol* 56, 289-314.

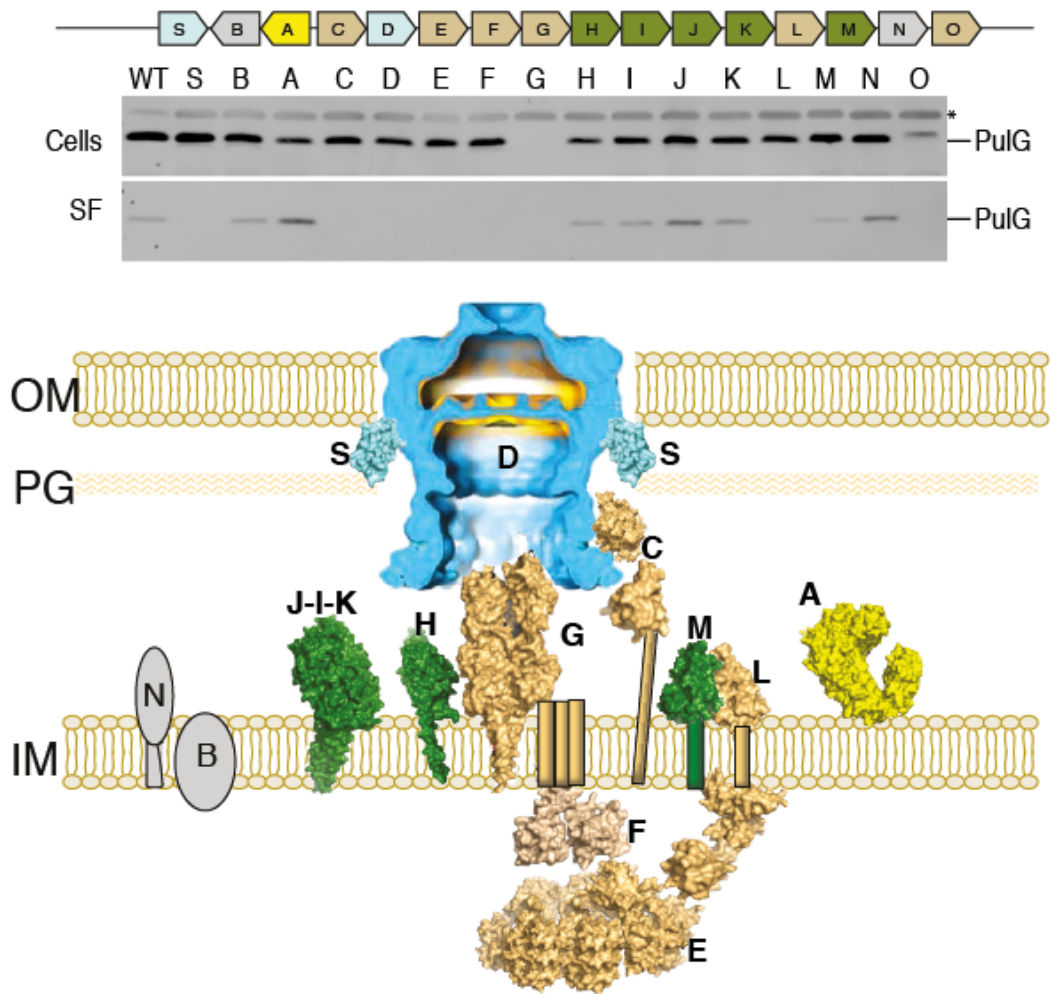
- 536 McLaughlin, L., R. Haft and F. KT, 2012. Structural insights into the Type II secretion
537 nanomachine. *Cu Op Struct Biol* 22, 208-216.
- 538 Mistic, A. M., K. A. Satyshur and K. T. Forest, 2010. *P. aeruginosa* PilT structure with and
539 without nucleotide reveal a dynamic type IV pilus retraction motor. *J Mol Biol* 400,
540 1011-1021.
- 541 Nunn, D., 1999. Bacterial type II protein export and pilus biogenesis: more than just
542 homologies. *Trends Cell Biol* 9, 402-408.
- 543 Patrick, M., K. V. Korotkov, W. G. Hol and M. Sandkvist, 2011. Oligomerization of EpsE
544 coordinates residues from multiple subunits. *J Biol. Chem* 286, 10378-10386.
- 545 Pelicic, V., 2008. Type IV pili: e pluribus unum? *Mol Microbiol* 68, 827-837.
- 546 Possot, O. M., G. Vignon, N. Bomchil, F. Ebel and A. P. Pugsley, 2000. Multiple
547 interactions between pullulanase secretion components involved in stabilization and
548 cytoplasmic membrane association of PulE. *J Bacteriol* 182, 2142-2152.
- 549 Pugsley, A. P., 1993. Processing and methylation of PulG, a pilin-like component of the
550 general secretory pathway of *Klebsiella oxytoca*. *Mol Microbiol* 9, 295-308.
- 551 Py, B., L. Loiseau and F. Barras, 2001. An inner membrane platform in the type II secretion
552 machinery of Gram-negative bacteria. *EMBO Rep* 2, 244-248.
- 553 Robien, M. A., B. E. Krumm, M. Sandkvist and W. G. Hol, 2003. Crystal structure of the
554 extracellular protein secretion NTPase EpsE of *Vibrio cholerae*. *J Mol. Biol* 333, 657-
555 674.
- 556 Satyshur, K. A., G. A. Worzalla, L. S. Meyer, E. K. Heiniger, K. G. Aukema, A. M. Mistic and
557 K. T. Forest, 2007. Crystal structures of the pilus retraction motor PilT suggest large
558 domain movements and subunit cooperation drive motility. *Structure* 15, 363-376
- 559 Sauvonnet, N., P. Gounon and A. P. Pugsley, 2000a. PpdD type IV pilin of *Escherichia coli* K-
560 12 can be assembled into pili in *Pseudomonas aeruginosa*. *J Bacteriol.* 182, 848-854.
- 561 Sauvonnet, N., G. Vignon, A. P. Pugsley and P. Gounon, 2000b. Pilus formation and protein
562 secretion by the same machinery in *Escherichia coli*. *EMBO J* 19, 2221- 2228.
- 563 Shevchik, V., J. Robert-Baudouy and G. Condemine, 1997. Specific interaction between OutD,
564 an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and
565 secreted proteins. *EMBO J* 16, 3007-3016.
- 566 Shiue, S. J., I.-L. Chien, N. L. Chan, W. M. Leu and N. T. Hu, 2007. Mutation of a key residue
567 in the type II secretion system ATPse uncouples ATP hydrolysis from protein
568 translocation. *Mol Microbiol* 65, 401-412.
- 569 Streif, S., W. F. Staudinger, W. Marwan and D. Oesterhelt, 2008. Flagella rotation in the
570 archaeon *Halobacterium salinarum* depends on ATP. *J Mol Biol* 384, 1-8.
- 571 Strom, M. S. and S. Lory, 1991. Amino acid substitutions in pilin of *Pseudomonas aeruginosa*.
572 Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. *J.*
573 *Biol. Chem.* 266, 1656-1664.
- 574 Vignon, G., R. Kohler, E. Larquet, S. Giroux, M. C. Prevost, P. Roux and A. P. Pugsley, 2003.
575 Type IV-like pili formed by the type II secretion: specificity, composition, bundling,
576 polar localization, and surface presentation of peptides. *J Bacteriol* 185, 3416-3428.
- 577 Wang, Y. A., X. Yu, S. Y. M. Ng, K. F. Jarrell and E. H. Egelman, 2008. The structure of an
578 archaeal pilus. *J Mol Biol* 381, 456-466.
- 579 Winther-Larsen, H. C., M. Wolfgang, S. Dunham, J. P. van Putten, D. Dorward, C. Lovold, F.
580 E. Aas and M. Koomey, 2005. A conserved set of pilin-like molecules controls type IV
581 pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. *Mol*
582 *Microbiol* 56, 903-917.
- 583 Wolfgang, M., J. P. M. van Putten, S. F. Hayes, D. Dorward and M. Koomey, 2000.
584 Components and dynamics of fiber formation define a ubiquitous biogenesis pathway
585 for bacterial pili. *EMBO. J.* 19, 6408-6418.

- 586 Yanez , M., K. Korotkov , J. Abendroth and W. G. J. Hol, 2008. The Crystal Structure of a B
587 inary Complex of two Pseudopilins: EpsI and EpsJ from the Type 2 Secretion System of
588 *Vibrio vulnificus*. J. Mol. Biol. 375, 471–486.
- 589 Yanez, M. E., K. V. Korotkov, J. Abendroth and W. G. Hol, 2008. Structure of the minor
590 pseudopilin EpsH from the Type 2 secretion system of *Vibrio cholerae*. J. Mol Biol
591 377, 91-103.
- 592 Yu, X., C. Goforth, C. Meyer, R. Reinhard, R. Wirth, G. F. Shchröder and E. H. Egelman,
593 2012. Filaments from *Ignicoccus hospitalis* show diversity of packing in proteins
594 containing N-terminal type IV pilin helices. J Mol Biol 422, 264-281.
- 595
596
597
- 598
599
600
601

602
603 **Figures**
604



605
606 **Fig. 1. Pseudopilus biogenesis steps.** The transmembrane segment of pseudopilins (in grey)
607 interacts with cellular and type II secretion system partners. The hydrophobicity of the segment
608 is crucial for the targeting *via* the SRP complex (A) to the Sec translocase (B) for correct
609 membrane insertion. Upon insertion, the pre-pilin peptidase cleaves the positively charged pre-
610 peptide after the conserved Gly residue (C); the cleaved peptide interacts with at least one
611 component of the assembly platform (D) to finally become buried in the core of the assembled
612 fiber, while the globular hydrophilic domains are exposed on the pseudopilus surface (E).
613
614
615
616
617
618



619

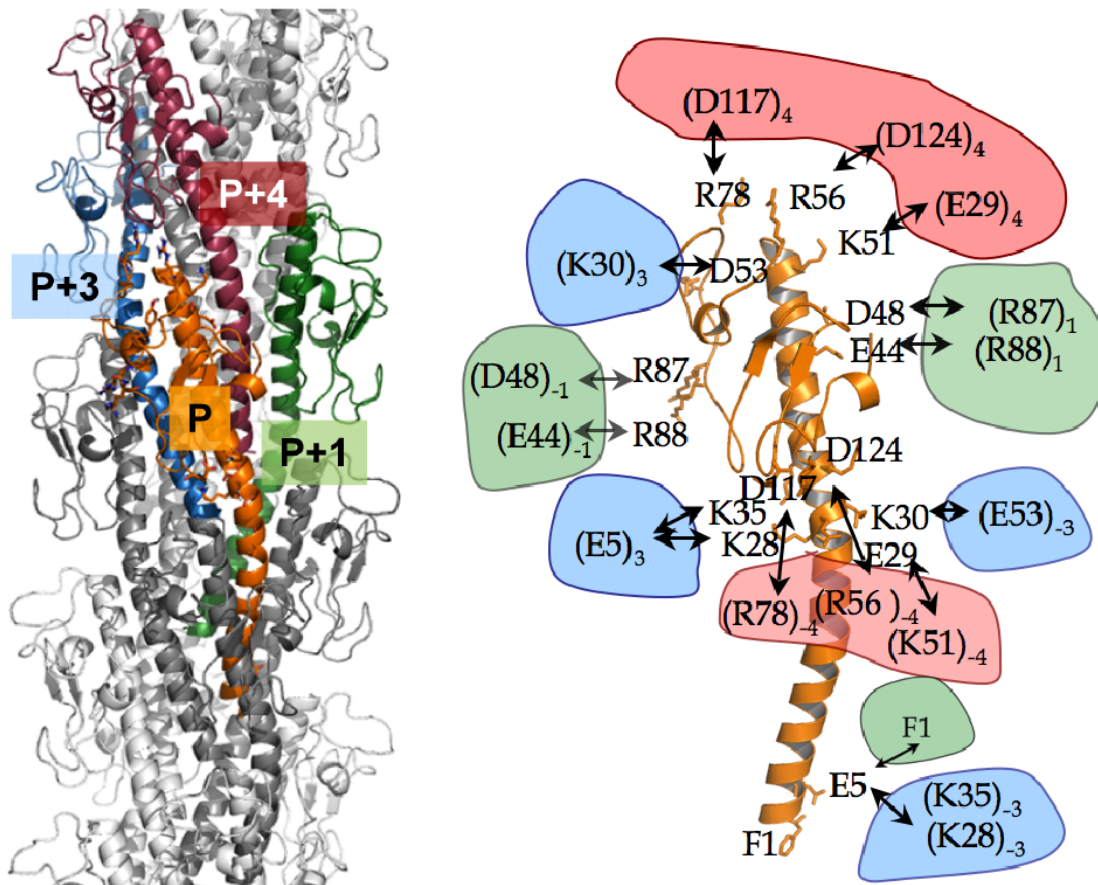
620 **Fig. 2. Involvement of T2SS components in pilus formation.** (A) Top: Organization of the
 621 Pul T2SS-encoding genes on the chromosome of *Klebsiella oxytoca*. Divergent operons *pulAB*
 622 and *pulCDEFGHIJKLMNO*, and a monocistronic *pulS* gene encode the T2SS. The substrate
 623 gene *A* is shown in yellow. Genes dispensable for the substrate secretion are shown in grey,
 624 genes essential for surface exposure of the pseudopilus and the substrate are in blue, genes
 625 essential for pseudopilus assembly and protein secretion are in pale orange, and genes essential
 626 for protein secretion only are in green. Below: PulG pilus assembly in mutants lacking
 627 individual T2SS components. Bacteria of strain MDS42 (Posfai et al., 2006) transformed with
 628 different derivatives of plasmid pCHAP231 carrying the complete set of pul genes or single
 629 nonpolar mutations as indicated (Possot et al., 2000) were grown for two days on rich solid
 630 media. Bacterial and sheared pili fractions were analyzed by immunodetection using anti-PulG
 631 antibodies, as described (Cisneros et al., 2011). The equivalent of 0.05 OD_{600nm} of each fraction
 632 was loaded. The asterisk indicates a cross-reacting band. (B) The schematic representation of
 633 the Pul T2SS components in the bacterial envelope with the same color- code as above. OM,
 634 outer membrane; PG, peptidoglycan; IM, inner membrane. Images of T2SS components were
 635 prepared with PyMol (<http://www.pymol.org>). The model of GspD is adapted from (Reichow
 636 et al., 2010).

637

638

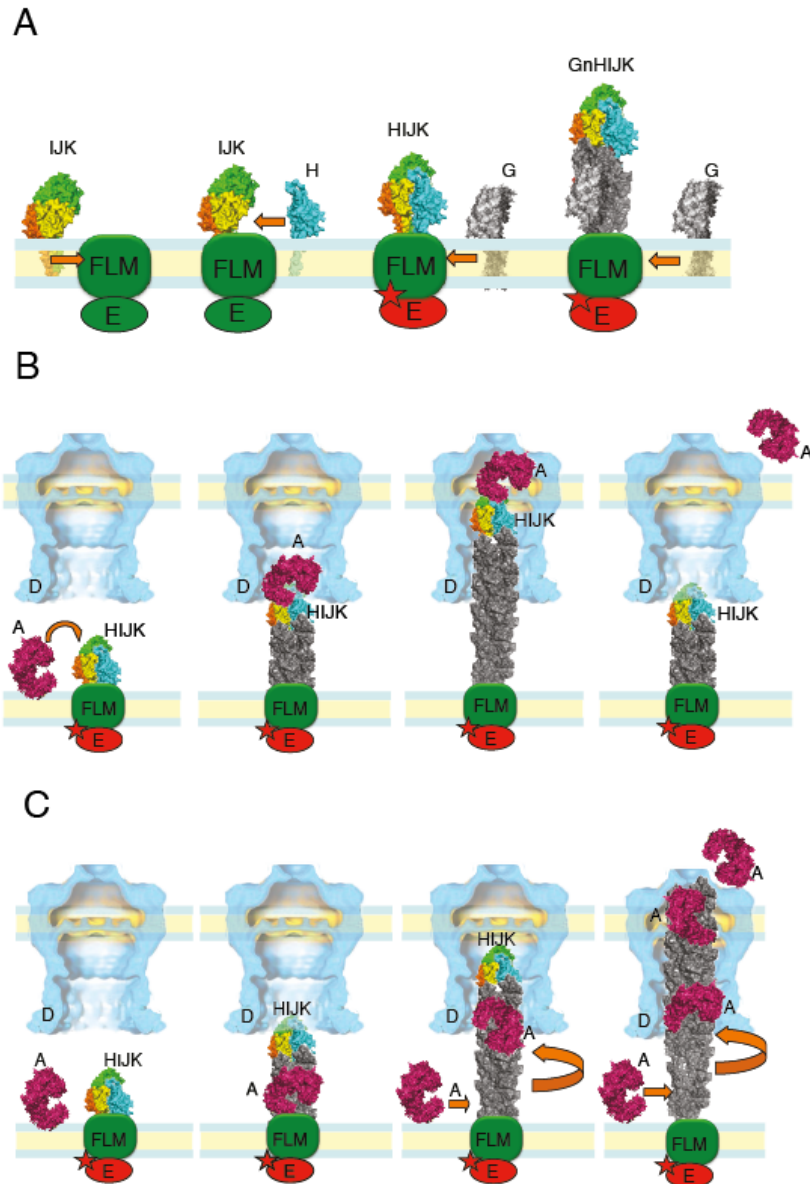
639

640



641
 642 **Fig. 3.** The structure of the PulG pseudopilus (left) and PulG residues involved in inter-
 643 protomer interactions (right). The central protomer P (orange) interacts with protomer P+1
 644 (green), P+3 (blue) and P+4 (red). Arrows indicate predicted interactions between residues of
 645 the central protomer P and its partners at each interface (indicated by the residue position, the
 646 color code and the protomer number in index). Adapted from (Campos et al., 2010).

647
 648
 649
 650
 651
 652



653

654 **Fig. 4. Models of pseudopilus assembly and function in protein secretion.** (A) The minor
 655 pseudopilins (I-J-K) self-assemble in the inner membrane (IM) and bind to the fourth minor
 656 pseudopilin GspH (H). This tetrameric complex transfers the activation signal to the assembly
 657 ATPase via an unknown component of the assembly platform, possibly GspL (LMF). The
 658 activation of the ATPase (E) leads to the elongation of the pseudopilus by successive addition
 659 of major pseudopilin subunits (G), coupled to the fiber rotation relative to the assembly
 660 ATPase. (B) According to the piston model, the substrate (A in pink) binds to the minor
 661 pseudopilin complex on the tip (HIJK). Pseudopilus elongation adding GspG subunits (in grey)
 662 pushes the substrate through the secretin channel. The system is reset by an unknown
 663 mechanism to allow binding of the next substrate molecule. (C) In the Archimedes' screw
 664 model, minor pseudopilin complex initiates pseudopilus assembly and the substrate binds to the
 665 major pseudopilins at the fiber base in the IM. Pseudopilus growth from the base, coupled with
 666 rotation drives the substrate into the secretin channel (D) vestibule. Pseudopilus assembly force
 667 pushes the secretin gate open and the substrate is expelled across the outer membrane (OM)
 668 channel. A single pseudopilus initiation event leads to secretion of multiple substrate molecules
 669 via continuous assembly of the major pseudopilins.