



**HAL**  
open science

## **Polar N-terminal Residues Conserved in Type 2 Secretion Pseudopilins Determine Subunit Targeting and Membrane Extraction Steps during Fibre Assembly**

Javier Santos-Moreno, Alexandra East, Ingrid Guilvout, Nathalie Nadeau, Peter J. Bond, Guy Tran van Nhieu, Olivera Francetic

► **To cite this version:**

Javier Santos-Moreno, Alexandra East, Ingrid Guilvout, Nathalie Nadeau, Peter J. Bond, et al.. Polar N-terminal Residues Conserved in Type 2 Secretion Pseudopilins Determine Subunit Targeting and Membrane Extraction Steps during Fibre Assembly. *Journal of Molecular Biology*, 2017, 429 (11), pp.1746 - 1765. 10.1016/j.jmb.2017.04.005 . pasteur-01602354

**HAL Id: pasteur-01602354**

**<https://pasteur.hal.science/pasteur-01602354>**

Submitted on 2 Oct 2017

**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.

1 Polar N-terminal residues conserved in type 2 secretion pseudopilins determine subunit  
2 targeting and membrane extraction steps during fibre assembly

3

4 Javier Santos-Moreno<sup>1-5#</sup>, Alexandra East<sup>6</sup>, Ingrid Guilvout<sup>7,8</sup>, Nathalie Nadeau<sup>7</sup>, Peter J.  
5 Bond<sup>9,10</sup>, Guy Tran Van Nhieu<sup>2-5</sup> and Olivera Francetic<sup>7,8\*</sup>

6

7 <sup>1</sup>Université Paris Diderot (Paris 7) Sorbonne Paris Cité

8 <sup>2</sup>Laboratory of Intercellular Communication and Microbial Infections, CIRB, Collège de  
9 France, Paris, France

10 <sup>3</sup>Institut National de la Santé et de la Recherche Médicale (Inserm) U1050, France

11 <sup>4</sup>Centre National de la Recherche Scientifique (CNRS), UMR7241, France

12 <sup>5</sup>MEMOLIFE Laboratory of Excellence and Paris Sciences et Lettres, France

13 <sup>6</sup>Centre for Molecular Informatics, Department of Chemistry, University of Cambridge,  
14 Lensfield Road, Cambridge, CB2 1EW, UK

15 <sup>7</sup>Laboratory of Macromolecular Systems and Signalling, Institut Pasteur, Department of  
16 Microbiology and CNRS ERL6002, 25 rue du Dr Roux, 75724 Paris, Cedex 15, France

17 <sup>8</sup>Biochemistry of Macromolecular Interactions Unit, Department of Structural Biology and  
18 Chemistry, 28 rue du Dr Roux, 75724 Paris, Cedex 15, France

19 <sup>9</sup>Bioinformatics Institute (A\*STAR), 30 Biopolis Str, #07-01 Matrix, Singapore 138671

20 <sup>10</sup> National University of Singapore, Department of Biological Sciences, 14 Science Drive 4,  
21 Singapore 117543

22

23 <sup>#</sup>Current address: Department of Fundamental Microbiology, University of Lausanne,  
24 Biophore building, CH1015 Lausanne, Switzerland.

25

26 <sup>\*</sup>Correspondence:

27 Institut Pasteur, 25 rue du Dr Roux, 75724 Paris CEDEX 15, France

28 email: ofrancet@pasteur.fr

29 Tel: 33 1 40 61 36 81

30 Fax: +33 1 45 68 89 60

31 Key words: type 4 fibre assembly, membrane proteins, immunofluorescence microscopy,  
32 molecular dynamics simulations, N-methylation

33

34 **Abstract**

35

36 Bacterial type 2 secretion systems (T2SS), type 4 pili (T4P) and archaeal flagella assemble  
37 fibres from initially membrane-embedded pseudopilin and pilin subunits. Fibre subunits are  
38 made as precursors with positively charged N-terminal anchors, whose cleavage *via* the  
39 prepilin peptidase, essential for pilin membrane extraction and assembly, is followed by N-  
40 methylation of the mature (pseudo)pilin N-terminus. The conserved Glu residue at position 5  
41 (E5) of mature (pseudo)pilins is essential for assembly. Unlike T4 pilins, where E5 residue  
42 substitutions also abolish N-methylation, the E5A variant of T2SS pseudopilin PulG remains  
43 N-methylated, but is affected in interaction with the T2SS component PulM. Here,  
44 biochemical and functional analyses showed that the PulM interaction defect only partly  
45 accounts for the PulG<sup>E5A</sup> assembly defect. First, PulG<sup>T2A</sup> variant, equally defective in PulM  
46 interaction, remained partially functional. Furthermore, pseudopilus assembly defect of  
47 *pulG(E5A)* mutant was stronger than that of the *pulM* deletion mutant. To understand the  
48 dominant effect of E5A mutation, we used molecular dynamics simulations of PulG<sup>E5A</sup>,  
49 methylated PulG<sup>WT</sup> (MePulG<sup>WT</sup>) and MePulG<sup>E5A</sup> variant in a model membrane. These  
50 simulations pointed to a key role for an intra-molecular interaction between the pseudopilin  
51 N-terminal amine and E5 to limit polar interactions with membrane phospholipids. N-  
52 methylation of the N-terminal amine further limited its interactions with phospholipid head-  
53 groups to facilitate pseudopilin membrane escape. By binding to polar residues in the  
54 conserved N-terminal region of PulG, we propose that PulM acts as chaperone to promote  
55 pseudopilin recruitment and coordinate its membrane extraction with subsequent steps of the  
56 fibre assembly process.

57

58

## 59 **Introduction**

60

61 Prokaryotes build diverse surface appendages and protein transport systems to colonize their  
62 niche and acquire nutrients. Some of the most ancient and versatile prokaryotic nanomachines  
63 that mediate these functions belong to the type 4 filament (Tff) superfamily <sup>1</sup>. These  
64 conserved membrane complexes use the ATP-derived energy to drive assembly of flagella  
65 (archaella) and pili in archaea <sup>2; 3; 4</sup> and to build T4P and T2SS pseudopili in bacteria.  
66 Illustrating the diversity of Tff functions, T4P, thin bacterial surface fibres, mediate  
67 adherence, aggregation, motility, protein transport and DNA uptake <sup>1; 5</sup>. In T2SSs, found in  
68 Gram-negative (or diderm) bacteria, short periplasmic pseudopilus fibres promote protein  
69 transport from the periplasm across the outer membrane (OM) <sup>6; 7; 8</sup>.

70 Bacterial Tffs are helical polymers of repeating subunits of the major pilin or  
71 pseudopilin, which may also contain one or more minor subunits that modulate the fibre  
72 assembly and function <sup>1</sup>. These subunits are made as membrane-embedded precursors, with  
73 an N-terminal cytoplasmic pre-peptide, followed by a highly conserved transmembrane  
74 segment (TMS) and a variable periplasmic globular domain that determines surface features  
75 of assembled fibres. The prepilin peptidase, an integral membrane aspartic protease, cleaves  
76 the positively charged peptide anchor on the cytoplasmic face of the TMS <sup>9</sup>. Bacterial prepilin  
77 peptidases have an additional, methyl-transferase domain that modifies the N-terminus of  
78 mature (pseudo)pilins; however the function of this N-methylation remains unclear <sup>10; 11</sup>.

79 In Gram-negative bacteria, Tff assembly systems form large envelope-spanning  
80 complexes that have been visualised recently for T4P by cryo-electron tomography <sup>12</sup>. Their  
81 most conserved parts are the cytoplasmic hexameric ATPase of the GspE family and the inner  
82 membrane (IM) platform protein of the GspF family <sup>13</sup>. Together with GspL and GspM, they

83 form the assembly platform (AP)<sup>14</sup>, which connects *via* the IM protein GspC with the GspD  
84 multimer forming the outer membrane secretin channel<sup>15</sup>.

85 In the T2SS of *Klebsiella*, dedicated to secretion of pullulanase (PulA),  
86 overproduction of the major pseudopilin PulG leads to assembly of fibres on the cell surface  
87 in plate-grown bacteria<sup>16</sup>. A similar phenomenon has been observed for the *Pseudomonas*  
88 T2SSs called Xcp<sup>17</sup>. Fibre assembly in overexpression conditions globally correlates with the  
89 ability of these systems to promote protein transport under physiological conditions,  
90 suggesting a mechanistic link between these two functions<sup>16</sup>. Some interactions of the T2SS  
91 major pseudopilin with assembly components have been recently unveiled. The PulG  
92 homologue in *Vibrio cholerae*, EpsG, forms a cross-linked heterodimer with EpsL, even in  
93 the absence of other Eps components<sup>18</sup>. EpsL is a bitopic IM protein that binds the ATPase  
94 EpsE *via* its cytoplasmic domain<sup>19; 20; 21</sup> and has been proposed to couple ATP hydrolysis to  
95 major pseudopilin polymerization<sup>18</sup>. Direct interaction of PulL and PulM T2SS homologues  
96 in different bacteria leads to their mutual stabilisation<sup>19; 22; 23; 24</sup>. Recent studies using  
97 bacterial two-hybrid (BAC2H) and co-purification approaches have shown that PulG interacts  
98 individually with PulM and PulF components of the inner membrane assembly platform<sup>25</sup>.  
99 Binding of major pilins to PulF, PulL and PulM homologues has also been demonstrated in  
100 the T4P assembly system of *Neisseria meningitidis*<sup>26</sup>, *Thermus thermophilus*<sup>27</sup> and  
101 *Pseudomonas aeruginosa*<sup>28</sup>, further supporting the direct role of AP in fibre assembly.

102 In the *P. aeruginosa* and *N. gonorrhoeae* major T4 pilin subunits, residue E5 is  
103 essential for pilus assembly and N-methylation<sup>9; 29; 30</sup>. In contrast, PulG variants with E5A or  
104 E5V substitutions are still N-methylated<sup>25; 31</sup>, nevertheless they are fully deficient in  
105 pseudopilus assembly and protein secretion, suggesting that this mutation affects a key step in  
106 the process<sup>31; 32; 33; 34</sup>. Recently we showed that residue E5 of PulG is a key determinant of  
107 PulG ⋈ PulM interaction in the absence of the other T2SS components<sup>25</sup>. Here, we studied the

108 role of this interaction and PulM function in the context of the T2SS. Since the defective  
109 interaction with PulM could not fully explain the loss of function of PulG<sup>E5A</sup> variant, we used  
110 molecular dynamics (MD) simulations to study PulG interactions with model membranes.  
111 The results suggested that both E5 and N-methylation reduce polar interactions of pseudopilin  
112 N-terminus with membrane phospholipids, revealing an essential stage of Tff assembly.

113

## 114 **Results**

115

### 116 *PulM requirement for PulA secretion and pseudopilus assembly*

117

118 Recent studies have identified the AP protein PulM as a strong interacting partner of PulG<sup>25</sup>.  
119 PulM is a bitopic IM component of the AP that stabilizes PulL, which provides the membrane  
120 anchor to the ATPase PulE<sup>19; 22; 23; 24</sup>. Unlike its homologues from *Vibrio* or *Pseudomonas*  
121 T2SSs, previous studies showed that PulM is only partially required for PulA secretion in  
122 strains overexpressing *pul* genes<sup>24</sup>, although it was essential for assembly of PulG pili<sup>16</sup>.  
123 Since in the past the roles of some factors involved in secretion had been overlooked due to  
124 overexpression, we reinvestigated the role of PulM in PulA secretion under conditions where  
125 *pul* genes were expressed at near-physiological levels, in *Escherichia coli* strain PAP5199  
126 carrying the *pcnB* mutation that reduces the copy number of expression plasmids (Materials  
127 and Methods).

128 At near-physiological expression levels, virtually all PulA was secreted in a strain  
129 producing all Pul T2SS components (Fig. 1A, WT lanes 5, 6; Fig. 1C) and secretion was  
130 abolished in the negative control strains lacking the PulE ATPase or PulG (Fig. 1A, lanes 1-4;  
131 Fig. 1C). A newly constructed mutant strain carrying a complete *pulM* deletion (plasmid  
132 pCHAP8496, Table 1 and Materials and Methods) had the same phenotype (Fig. 1A, lanes 7,

133 8; Fig. 1C), showing that PulM is essential for PulA secretion under physiological conditions.  
134 As PulM is involved in PullL stabilization, we asked whether function could be restored by co-  
135 expressing an extra copy of *pullL* from a P<sub>lacZ</sub> promoter in a compatible plasmid (Fig. 1A,  
136 lanes 9, 10; Fig. 1C). However, increased PullL levels did not improve PulA secretion,  
137 suggesting that PulM might play an additional role. The secretion defect of the  $\Delta pulM$  mutant  
138 was complemented with *pulM* or *pullLM* expressed *in trans* (Fig. 1A, lanes 13-16; Fig. 1C).

139 The Pul T2SS is able to assemble PulG into fibres on the bacterial surface, when the  
140 *pul* genes are overexpressed from a moderate copy-number plasmid in bacteria cultured on  
141 LB agar media<sup>16</sup>. Here, using the new  $\Delta pulM$  allele, we re-assessed the role of PulM in  
142 piliation (Materials and Methods) (Fig. 1B). PulG fibres were assembled in a manner fully  
143 dependent on PulE ATPase (Fig. 1B, lanes 1, 2 and 5, 6; Fig. 1D), while no signal was  
144 observed in the control lacking PulG (Fig. 1B, lanes 3, 4; Fig. 1D). The absence of PulM led  
145 to a severe defect, but did not fully abolish piliation (Fig. 1B, lanes 7, 8; Fig. 1D), confirming  
146 our previous observations<sup>7</sup>. In contrast, PullL, which recruits PulE to the AP, was essential for  
147 piliation (Fig. 1B, lanes 11, 12). Piliation in the  $\Delta pulM$  strain was restored by  
148 complementation with *pulM* (Fig. 1B, lanes 13, 14; Fig. 1D), while overproduction of PullL  
149 exacerbated the piliation defects in  $\Delta pulM + pullL$  and  $\Delta pulM + pullLpulM$  strains (Fig. 1B,  
150 compare lanes 7, 8 with 9, 10 and 13, 14 with 15, 16; Fig. 1D), indicating that assembly of  
151 surface pili requires the correct ratio between these Pul components. PulM was destabilized in  
152 the absence of PullL, and the reverse was also true (Fig. 1B) confirming previous observations  
153<sup>24</sup>. Together, these results show that PulM is essential for PulA secretion under physiological  
154 expression conditions and that PulG pilus assembly, although very inefficient, was not fully  
155 abolished in the  $\Delta pulM$  mutant.

156

157 *Effect of pul gene overexpression on piliation and secretion in the  $\Delta pulM$  mutants*

158 To further characterize the *ΔpulM* mutant, we compared its piliation defect to that of  
159 the previously characterized *Δpull* strain, which lacks PulI, one of the three minor  
160 pseudopilins involved in assembly initiation<sup>35</sup>. As expected, the *ΔpulM* mutant was more  
161 deficient in pilus assembly compared to the *Δpull* strain (Figs. 2A and B). Analysis of the  
162 same strains in liquid culture conditions showed that *pul* gene overexpression did not improve  
163 PulA secretion in *ΔpulE*, *ΔpulG* and *Δpull* mutants. However, more than 10% of PulA was  
164 secreted in the *ΔpulM* mutant (Fig. 2C and 2D), confirming previous findings<sup>24</sup> and  
165 suggesting that overproduction of another Pul component might compensate for the absence  
166 of PulM.

167 To test whether PulG overproduction allows to overcome this defect, we transformed  
168 the *pul* mutant strains used above with plasmid pCHAP8568 (Table 1) carrying *pulG* under  
169 the control of the *lacZ* promoter. While increased PulG levels did not overcome the  
170 requirement for the PulE ATPase, piliation was improved in *Δpull* + *pulG* to an extent  
171 comparable to that observed for the positive control (*ΔpulG* + *pulG*) (Fig. 2E and F). In  
172 contrast, PulG overproduction led only to a marginal increase of PulG in the sheared fraction  
173 of the *ΔpulM* mutant, suggesting that PulM is nearly essential, either for T2SS assembly and  
174 integrity or for its activity.

175

176 *Analysis of pulM phenotype by immunofluorescence (IF) microscopy*

177

178 To assess in more detail the piliation defect of the *ΔpulM* strain, we analysed samples  
179 of plate-grown bacteria using IF microscopy and anti-PulG antibodies (Materials and  
180 Methods). PulG fibres were detected in strain producing the complete Pul T2SS (WT) and not  
181 in the negative controls lacking the PulE ATPase or PulG (Fig. 3A, *ΔE* and *ΔG*). Consistent  
182 with the results of the shearing assay, fewer extracellular fibres were detected in the absence



183 of PulM (Fig. 3A,  $\Delta M$ ) compared to the  $\Delta pull$  mutant (Fig. 3A,  $\Delta I$ ) characterised previously  
184 <sup>35</sup>. While WT strain produced on average  $0.47 \pm 0.09$  fibres per bacterium, the  $\Delta pull$  and  
185  $\Delta pulM$  strains produced on average 4-fold and 52-fold fewer fibres per bacterium,  
186 respectively (Fig. 3B). The measurements of the length (Y-axis) of individual fibres (dots)  
187 show that, compared to WT strain, the  $\Delta pull$  and  $\Delta pulM$  strains produced longer fibres on  
188 average (Fig. 3B). Plotting the relative frequency distributions of the fibre length in WT and  
189  $\Delta pull$  and  $\Delta pulM$  mutants further illustrates this tendency (Fig. 3C).

190 The increased PulG levels led to an increase in fibre numbers and median length to a  
191 similar extent in all strains (Fig. 4A, B and D). However, while PulG overproduction restored  
192 piliation in  $\Delta pulG$  and  $\Delta pull$  strains, in the  $\Delta pulM$  mutant strain, the piliation defect remained  
193 dramatic with only ~10% of PulG found in the sheared fraction (Fig. 2E).

194

195 *Differential effect of PulG<sup>E5A</sup> and PulG<sup>T2A</sup> on pseudopilus assembly and PulA secretion*

196

197 We showed recently that E5A substitution in PulG strongly affects PulM binding,  
198 raising a possibility that functional defects of PulG<sup>E5A</sup> could be due to the effect on PulG-  
199 PulM interaction <sup>25</sup>. Residue E5 is localized in the highly conserved N-terminal TMS of major  
200 and minor pseudopilins <sup>33</sup>. Another highly conserved polar residue in this segment of mature  
201 pseudopilins and T4 pilins is Thr at position 2 (T2) (Fig. 5A). Interestingly, T2 and E5 show a  
202 degree of co-variation when different pseudopilin sequences are aligned (Fig. 5B). This is  
203 linked to the absence of E5 in the GspK family of T2SS pseudopilins and to the conservation  
204 of A2 residue in these proteins (Fig. S1). To further characterize this conserved N-terminal  
205 region of PulG, we generated the PulG<sup>T2A</sup> variant and tested its interaction with PulM using  
206 the BAC2H approach <sup>36</sup>. Full-length PulM and mature PulG or its variants fused to the C-  
207 terminus of T18 and T25 fragments of the catalytic domain of the CyaA adenylyl cyclase

208 from *Bordetella pertussis* were co-produced in an *E. coli*  $\Delta$ *cya* mutant strain DHT1<sup>37</sup>. Fusion  
209 protein interactions resulted in functional complementation and conversion of ATP into  
210 cAMP, monitored using the *lacZ* gene as a reporter.

211 The high  $\beta$ -galactosidase activity of strains co-producing PulM- and PulG- hybrids  
212 indicated their strong interaction (Fig. 5C), which was negatively affected by the E5A  
213 substitution (Fig. 5C)<sup>25</sup>. Similarly, the T2A substitution in PulG led to reduced *lacZ*  
214 expression, showing that T2 is also important for PulM-PulG interaction. All these PulG  
215 variants were still able to form homo-dimers (Fig. 5C), which is in agreement with the  
216 evidence that residues in the globular head, but not in the N-terminal TMS, drive PulG  
217 dimerization<sup>34</sup>. As a control of specificity, we substituted another highly conserved residue of  
218 PulG TMS, proline at position 22 (P22), by an alanine. The  $\beta$ -galactosidase activity of strains  
219 co-producing PulM- and PulG<sup>P22A</sup>- chimera did not differ significantly from that of strains  
220 producing the wild type PulM- and PulG- hybrids, showing a specific role of conserved  
221 residues T2 and E5 in PulG interaction with PulM.

222 We next tested the impact of the T2A substitution on PulG function. A  $\Delta$ *pulG* strain  
223 complemented with the *pulG(T2A)* allele showed a ~50% reduced ability to assemble T2SS  
224 pili (Fig. 5D and 5E). Likewise, secretion of PulA was reduced by ~50% for the PulG<sup>T2A</sup>  
225 variant, when all of the *pul* genes were co-expressed at physiological levels (Fig. 5F and 5G),  
226 showing a phenotype strikingly different from *pulG(E5A)* mutants.

227 Analysis by IF microscopy showed similar trends. In strain carrying *pulG(T2A)* allele,  
228 two-fold fewer fibres were observed relative to *pulG*<sup>WT</sup>, and the defect was less severe than  
229 for  $\Delta$ *pulM* + *pulG* and  $\Delta$ *pulG* + *pulG(E5A)* (Fig. 4A). Surprisingly, surface fibres in  $\Delta$ *pulG* +  
230 *pulG(T2A)* strain were shorter on average than those of the positive control  $\Delta$ *pulG* + *pulG*  
231 (Fig. 4A, C and E). Although small, this difference is highly statistically significant and raises

232 the possibility that the T2A mutation might also affect fibre elongation, for example by  
233 impairing recruitment of PulG subunits to the AP.

234

235 *PulG interaction with PulM in the context of the Pul T2SS*

236

237 Despite their functional differences, in the BAC2H assay the PulG<sup>T2A</sup> and PulG<sup>E5A</sup>  
238 variants were similarly impaired in interaction with PulM. To test whether PulG and PulG<sup>E5A</sup>  
239 interacted with PulM in the context of the complete T2SS, we used a cross-linking approach.  
240 *E. coli* PAP7460 bacteria moderately overproducing the Pul T2SSs and different PulG  
241 variants were treated with 0.6% formaldehyde (FA), and complexes containing PulM were  
242 detected using anti-PulM antibodies (Materials and Methods). In addition to PulM monomers  
243 and homo-dimers, we observed a ~38 kDa band that corresponds to a PulG-PulM heterodimer  
244 (Fig. 6, G-M), as it was only present in strains producing PulG (Fig. 6). Further supporting its  
245 identity as a G-M heterodimer, this band showed shifts in migration dependent on the  
246 molecular mass of co-produced PulG and its variants containing a C-terminal hexahistidine  
247 tag (Fig. 6, lanes 3, 4) or containing, in addition, the 6-residue long N-terminal pre-peptide (in  
248 strain lacking PulO, Fig. 6, lane 2). The levels of PulM-PulG<sup>E5A</sup>-His<sub>6</sub> heterodimer were  
249 reduced relative to PulM-PulG-His<sub>6</sub>. However, consistent with the PulM-PulG interaction  
250 study<sup>25</sup>, the E5A substitution in PulG did not fully abolish its interaction with PulM in the  
251 context of the T2SS, failing to fully account for its dramatic effect on PulG assembly and  
252 function.

253

254 *Molecular dynamics simulations of PulG and its variants in POPE model membrane*

255

256 Compared to PulG<sup>WT</sup>, variant PulG<sup>E5A</sup> is more stable<sup>34</sup> and accumulates in bacteria to  
257 higher levels, even in the absence of other T2SS components<sup>25</sup>. We therefore hypothesized  
258 that PulG<sup>E5A</sup> might be blocked in the membrane prior to entry into the assembly pathway. To  
259 test this possibility, we used atomistic MD simulations of PulG in model membranes *in silico*.  
260 This approach allowed us to study conformations and dynamics of PulG in its native  
261 environment and to quantify its atomic contacts with the membrane and solvent. Following  
262 cleavage by prepilin peptidase, PulG is N-terminally methylated at the conserved Phe residue  
263 (hereafter designated MeF1)<sup>10</sup>. Previous analyses using mass spectrometry showed that most  
264 of PulG<sup>E5A</sup> was methylated whereas ~30% of PulG<sup>E5A</sup> that co-assembled with PulG<sup>WT</sup> into  
265 mixed pili remained non-methylated<sup>25</sup>. To compare all these forms of PulG, we embedded  
266 structural models of a methylated MePulG<sup>WT</sup>, as well as those of methylated MePulG<sup>E5A</sup> and  
267 non-methylated PulG<sup>E5A</sup> monomers, into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-  
268 phosphoethanolamine (POPE) model membranes, and performed independent triplicate 200-  
269 350 ns MD simulations of each.

270 Initial and representative final snapshots of these simulations (Fig. 7) show that, in the  
271 final snapshot, the protein TMS became more deeply buried and its head domain bent towards  
272 the lipid membrane surface to varying degrees. The exception was one of the PulG<sup>E5A</sup>  
273 simulations, wherein the bottom of the globular domain nevertheless interacted extensively  
274 with the lipid by the end of the simulation. The solvent-accessible surface area (SASA) of  
275 residues 60-125, the bulk of the globular domain, decreased on average between ~0.2 and  
276 ~3.1 nm<sup>2</sup> over the course of the simulations. Overall the MD simulations did not reveal any  
277 statistically significant variations in protein conformation between variants, and SASA  
278 suggested that the globular domain does not maintain strong, consistent contacts with POPE.  
279 As expected, the E5A variants did not cause severe protein destabilization or membrane  
280 perturbation, supporting the notion that the role of E5 is localized to the N-terminus.

281           Next, we compared the maintenance of the protein secondary structure during each  
282 simulation. In all the systems, the N-terminal  $\alpha$ -helix, with the exception of the first 4  
283 residues, was almost entirely conserved throughout the simulations, as were the two  $\beta$ -sheets  
284 between residues 97-102 and 110-114. Transient disruptions to the  $\alpha$ -helix were observed in  
285 one PulG<sup>E5A</sup> simulation (with residues 30-34, or solely residue 34, losing their helical  
286 structure) and in one MePulG<sup>WT</sup> simulation (where the helix disintegrated around either  
287 residues 27-30 or again around residue 34). However, helix integrity was fully restored by the  
288 end of these simulations. On the other hand, we observed significant differences in the  
289 secondary structure of the five N-terminal residues between wild type and E5A variants. In all  
290 MePulG<sup>WT</sup> simulations the first several residues demonstrated a relaxation of helical structure  
291 into either turns or coils, as illustrated in Figure 8. In contrast, in the MePulG<sup>E5A</sup> and PulG<sup>E5A</sup>  
292 systems, the five N-terminal residues maintained their helical structure throughout, except in  
293 one Me-PulG<sup>E5A</sup> simulation, where six N-terminal residues transiently formed a 3-10 helix or  
294 turn structure between ~150-200 ns. Together, these results suggested that the residue E5  
295 affects the secondary structure of the PulG N-terminus.

296           To study this phenomenon more closely, we quantified the proximity of E5 to the N-  
297 terminus in all simulations. The distance between the centres-of-mass (COM) of residues 1  
298 and 5 was largely fixed due to the helix on which they are both located. However, significant  
299 differences were observed in the minimum distance between the residue 1 methyl group or  
300 amide terminus (depending on whether the system contained MeF1 or F1) and any atom of  
301 the residue 5 side-chain (Fig. 9A). Notably, in the MePulG<sup>WT</sup> system the methyl group of  
302 MeF1 and the E5 side-chain remained consistently within 3 Å of each other, indicating their  
303 electrostatic contact. This was not the case in the simulations of PulG variants carrying the  
304 E5A substitution. In the PulG<sup>E5A</sup> system, the A5 side-chain made no contact with F1 and  
305 remained at 6 Å distance, only approaching to within 5 Å in two simulations, and solely in the

306 first 30 or 70 ns. The MePulG<sup>E5A</sup> simulations showed more fluctuation, with the atoms  
307 approaching to within ~2.5 Å and moving as far as 10 Å apart; however, the side-chains  
308 mostly remained ~6 Å apart. Visual analysis shown in Fig. 9B supported these results; E5 and  
309 MeF1 remained close in MePulG<sup>WT</sup>, while in the MePulG<sup>E5A</sup> system A5 stayed embedded in  
310 lipid whereas MeF1 was extensively solvated. Importantly, in MePulG<sup>WT</sup> the N-terminal  
311 amine was engaged in interaction with E5 and did not form polar contacts with POPE  
312 phosphate groups, while in PulG<sup>E5A</sup> it was anchored to three different POPE molecules  
313 towards the end of the simulation (Fig. 9B). The MePulG<sup>E5A</sup> showed an intermediate  
314 behaviour, with reduced amine interactions with POPE, perhaps explaining the fluctuating  
315 distances to A5 during MD simulations. These data suggest that in MePulG<sup>WT</sup> E5 promotes  
316 intra-molecular interactions with the N-terminal positive charge of MeF1, thus reducing the  
317 polar contacts with phospholipid head-groups, and potentially priming the protein for  
318 membrane extraction during pseudopilus assembly. In contrast, the N-termini of the mutant  
319 variants interacted with membrane phospholipids, anchoring the protein more firmly in the  
320 bilayer.

321         Relative to the mutant systems, the MePulG<sup>WT</sup> experienced extended periods with no  
322 hydrogen bonds to solvent water, and its MeF1 formed 0.6 hydrogen bonds to water, on  
323 average. The MeF1 from MePulG<sup>WT</sup> and MePulG<sup>E5A</sup> proteins formed on average ~0.9  
324 hydrogen bonds with POPE, relative to the ~1.9 hydrogen bonds formed by F1 from non-  
325 methylated PulG<sup>E5A</sup>. When normalised relative to the number of possible hydrogen bonds  
326 from each, this analysis demonstrated that F1 engaged in hydrogen bonding to phospholipid  
327 40% more than MeF1. The methylation therefore decreased the number of hydrogen bonds to  
328 phospholipid head-groups, which might reduce the energetic cost of transferring the charged  
329 N-terminus across the IM.

330 T2 residue dynamics were also explored. T2 in MePulG<sup>WT</sup> formed only one hydrogen  
331 bond to E5 during a single simulation frame, and only sporadic hydrogen bonds were  
332 observed between the T2 hydroxyl oxygen and the MeF1 benzene ring. Instead, T2 formed on  
333 average ~1.4 hydrogen bonds with water and ~0.9 hydrogen bonds with surrounding POPE  
334 molecules. Likewise, in the MePulG<sup>E5A</sup> variant, T2 formed on average ~1.3 and ~0.8  
335 hydrogen bonds to water and to POPE respectively, and did not form any bonds with MeF1.  
336 Altogether, the simulations suggested that in the wild type, E5 engages in interaction with  
337 MeF1, leaving T2 available for interactions with solvent, membrane or other proteins,  
338 possibly PulM. The variation of residue 5 did not have a significant effect on T2 dynamics.

339

## 340 **Discussion**

341

342 To elucidate the early steps of pseudopilus assembly, we focused in this study on the  
343 interactions of the major pseudopilin PulG with the PulM AP component of the *K. oxytoca*  
344 T2SS. The results showed that PulM is fully required for PulA secretion under physiological  
345 conditions. On the other hand, overproduction of T2SS components resulted in dramatically  
346 reduced (but not fully abolished) PulA secretion and PulG fibre assembly in the *pulM*  
347 knockout. This is in agreement with results obtained in *P. aeruginosa* lacking its homologue  
348 XcpZ, which also shows low levels of piliation<sup>38</sup>. Quantitative IF microscopy analysis  
349 indicated that *pulM* mutants assemble at least 50-fold less pili compared to wild type. This  
350 defect is probably underestimated, since only fields containing at least one pilus were taken  
351 into account during IF quantification (Materials and Methods). Contrary to our predictions,  
352 the fibre length was not reduced but rather increased in  $\Delta pulM$  mutants.

353 The fact that overproduction of PulG could not restore efficient piliation in  $\Delta pulM$   
354 strains, together with reduced Pull levels is consistent with a requirement of PulM for

355 assembly of the functional T2SS complex<sup>25</sup>. The recent insights into the *Myxococcus xanthus*  
356 T4P machinery from cryo-electron tomography reveal a continuous, envelope-spanning  
357 apparatus<sup>12</sup>. Given the structural similarities between T4P and T2SSs, it is likely that the T2S  
358 nano-machine is organized in a similar manner, so that PulM, together with Pull, might  
359 constitute the lower periplasmic electron-dense ring in the periphery of the complex. In *M.*  
360 *xanthus*, this ring is absent in bacteria lacking PilO, which is a probable PulM equivalent  
361 sharing the same topology<sup>12</sup>. Studies of T2SS GFP chimera using live fluorescence  
362 microscopy support the localization of the *V. cholerae* PulM homologue EpsM in fluorescent  
363 foci dependent on the GspD, GspC and GspL homologues<sup>39</sup>. Since PulD-mCherry chimera  
364 form similar fluorescent foci in the absence of other Pul factors, the secretin assembly  
365 probably initiates the assembly of the T2SS complex<sup>40</sup>. These and other studies suggest that  
366 PulM might serve as a link bringing together the pseudopilin/PulF complex and the PulD/C/L  
367 complex through its interactions with PulG/PulH on one hand and Pull on the other<sup>19; 22; 23;</sup>  
368<sup>24; 25</sup>. Therefore, the observed critical role of PulM in fibre assembly could be due to its  
369 requirement for building a functional T2SS machine and its role in Pull stabilization,  
370 documented in several T2SSs<sup>19; 22; 23; 24</sup>. The rare PulG fibres that form in  $\Delta pulM$  mutants  
371 under overexpression conditions might stem from the few T2SS complexes that assemble  
372 through a low-affinity interaction of PulG with Pull<sup>25</sup>. The accumulation of unassembled  
373 PulG in the IM would drive elongation of pili within these rare T2SS complexes, thereby  
374 accounting for the higher median length of fibres in  $\Delta pulM$  mutants. A similar effect was  
375 observed previously for the mutants lacking minor pseudopilins of the tip complex, including  
376 Pull, used here as a control<sup>35</sup>.

377 In addition to the PulM role in Pull stabilization, its interaction with PulG in the  
378 absence of other Pul factors<sup>25</sup> suggested an additional, direct role in pseudopilus assembly.  
379 Here, using chemical cross-linking, we showed that PulM and PulG also interact in the



380 presence of other T2SS components, and that the E5A substitution reduced interaction with  
381 PulM. Both PulG and PulM are integral membrane proteins that share the N-in C-out  
382 topology. In addition to the PulG residue E5, we show here that residue T2 in this region is  
383 equally required for PulM interaction in the BAC2H assay, possibly accounting for residual  
384 PulM binding of the PulG<sup>E5A</sup> variant. This is also supported by MD simulation results, where  
385 residue T2 remained sterically available for inter-molecular interactions in all variants. The  
386 conformation of MePulG<sup>WT</sup> is compatible with PulM binding *via* T2 residue, which would  
387 explain the effects of PulG T2A substitution on PulM interaction and its impact on the fibre  
388 length. Furthermore, given that in MD simulations residue E5 of mature MePulG<sup>WT</sup> is  
389 engaged in interactions with the N-terminal amine, T2 might be an important determinant of  
390 this interaction during the membrane extraction step of PulG assembly. Since T2 formed  
391 hydrogen bonds with the surrounding solvent and membrane in all simulations, PulM binding  
392 might shield T2 and reduce these contacts to facilitate PulG membrane extraction, or its  
393 transfer to another binding partner. The high conservation of T2 and E5 among T2SS  
394 pseudopilins and T4 pilins (Fig. 5A) further shows the importance of PulG-PulM interface,  
395 whose disruption, at least on the PulG side, correlates with severe functional defects. Testing  
396 this model requires mutagenesis studies of the PulM cytoplasmic tail and/or TMS. Although  
397 no structural information on this part of PulM and its homologues is available, the predicted  
398 topology of these regions makes them obvious candidates for PulG binding.

399         Although the interaction with PulM was affected to a similar extent for PulG<sup>E5A</sup> and  
400 PulG<sup>T2A</sup> variants in the BAC2H assay, the functional impact of the two substitutions was very  
401 different. Compared to the E5A substitution that fully abolished function, the T2A  
402 substitution resulted in a partial defect in both fibre assembly and protein secretion (Fig. 5).  
403 The weaker PulM - PulG interaction reduced by half the number of assembled pili, which  
404 might directly reflect the number of assembled T2SS complexes. In addition, the pseudopili

405 composed of PulG<sup>T2A</sup> variant showed lower median length compared to wild type. This might  
406 reflect reduced fibre stability, in agreement with recent structural analysis of meningococcal  
407 T4P that have implicated residue T2 in inter-protomer interactions, involving residue E5 of  
408 the protomer below <sup>41</sup>. However, the defective PulA secretion in the presence of PulG<sup>T2A</sup>  
409 supports an early role of T2 in PulG assembly, consistent with previous studies showing that  
410 PulG pilus stability is not required for secretion <sup>33; 34</sup>. In this model, PulM binding might  
411 facilitate PulG recruitment to the machinery, e.g. by targeting PulG subunits to the active site  
412 of the AP. In addition, PulM might act as a chaperone, favouring the PulG conformation  
413 compatible with assembly. In *V. cholerae*, a cross-linking study showed direct interaction  
414 between the major pseudopilin EpsG and the PulL homologue EpsL <sup>18</sup>, and our recent study  
415 indicates weak interactions between PulL and PulG <sup>25</sup>. While the absence of prepilin  
416 peptidase in *V. cholerae* prevents the EpsG-EpsL cross-linking, in our studies the presence of  
417 the prepeptide seems to enhance PulG-PulM interaction, as suggested by the similar levels of  
418 heterodimers in the strain lacking PulO, which contained lower levels of PulG monomer (Fig.  
419 6, anti-G, lane 2). The preferential binding of precursor and mature forms of major  
420 pseudopilin to different partners in the AP - L or M - might reflect different conformations  
421 that represent intermediates in the assembly pathway.

422 To gain insight into these PulG conformations in wild type and E5A mutant variants  
423 we turned to MD simulations. Unexpectedly, this approach highlighted a role of another  
424 interacting partner of PulG - the plasma membrane, which poses a major obstacle to assembly  
425 of type 4 filaments in bacteria. By revealing the striking conformational difference between  
426 the N-termini of MePulG<sup>WT</sup> compared to MePulG<sup>E5A</sup> and PulG<sup>E5A</sup> variants in the membrane,  
427 the results of MD simulations provide a plausible explanation for the phenotypes of  
428 *pulG(E5A)* mutants. Contrary to the PulG<sup>E5A</sup> whose N-terminal amine is firmly anchored in  
429 the membrane through polar contacts with up to three different phospholipids, in MePulG<sup>WT</sup>

430 residue E5 neutralized the N-terminal membrane anchor. The T4P assembly models proposed  
431 by Craig *et al.*<sup>42</sup> and Melville and Craig<sup>5</sup> emphasise the importance of charge neutralisation  
432 during fibre assembly. While these models consider the inter-protomer charge neutralisation,  
433 the results of MD simulations and (Me)PulG<sup>E5A</sup> membrane accumulation strongly support the  
434 role of intra-molecular neutralisation of the N-terminal amine *via* a loop formation with E5.  
435 The intra-molecular contacts between F1 and E5 residues have also been observed in the  
436 crystal structure of T4 pilins of the *P. aeruginosa* PAK strain PilA (Protein database (PDB)  
437 entry code: 1OQW) and in *N. gonorrhoeae* pilin PilE (PDB: 2HI2)<sup>42; 43</sup>. A similar loop  
438 structure is suggested by the MD analyses of the *P. aeruginosa* major T4P subunit PilA<sup>44</sup>.  
439 Interestingly, in that study the loop is observed in initial stages of MD simulation, whereas  
440 towards the end, when PilA is fully embedded in the membrane, the N-terminal amine  
441 interacts with the phospholipid head-groups. The difference compared to our study is that  
442 PilA was not modelled with a methyl group, which might have led to ceasing of the intra-  
443 molecular F1-E5 interaction by the end of the simulation. Following the positive inside rule  
444<sup>45</sup>, the positively charged residues of IM proteins are anchored to the cytoplasmic face of the  
445 IM through interactions with negatively charged phosphate groups of membrane  
446 phospholipids. Besides, since the MD simulation conditions do not take into account the  
447 proton gradient, the membrane anchoring of MePulG<sup>WT</sup> and (Me)PulG<sup>E5A</sup> might be even  
448 stronger *in vivo* and might represent a rate-limiting step during pseudopilus assembly. Strong  
449 membrane association and stable N-terminal helix conformation could explain the decreased  
450 turnover rate of the PulG<sup>E5A</sup> compared to PulG<sup>WT</sup> as measured previously<sup>34</sup> and its relative  
451 accumulation in *E. coli*<sup>25</sup>.

452 The MD simulation results suggest that N-methylation might further reduce polar  
453 contacts of pilins with the solvent, contributing to their extraction from the membrane. Our  
454 observations using this approach also provide a clue for the link between E5 and N-

455 methylation, observed previously. While the E5K or E5V substitutions in the major subunit of  
456 *P. aeruginosa* T4P PilA abolish N-methylation<sup>9; 46</sup>, the E5A substitution in PulG only  
457 reduces the methylation efficiency by around 30%<sup>25</sup>. Enhanced N-terminal amine contacts  
458 with phospholipid head-groups in these variants might block or reduce the access of N-methyl  
459 transferase domain of prepilin peptidase to its amine substrate. The different effects of E5  
460 residue substitutions on T4 pilins and T2SS pseudopilins might be linked to the differences  
461 between their AP components such as PulM, a hypothesis that requires further investigation.

462         Among Tff systems, archaeal pili and flagella do not have the conserved residue E5<sup>47</sup>,  
463 and archaeal prepilin peptidases do not have a methyl-transferase domain and activity. One  
464 possible explanation for these differences might lie in the different composition of archaeal  
465 membranes, formed by tetraether lipids that are structurally and functionally different from  
466 bacterial phospholipids<sup>48</sup>. Rare exceptions to this rule include *Aeropyrum pernix* and  
467 *Archaeoglobus fulgidus*, where flagellins do have a conserved E5 residue, and whose  
468 presence correlates with the documented presence of phospholipids in their membranes<sup>49; 50</sup>.  
469 However, in most archaea the rules of membrane anchoring for proteins might be very  
470 different compared to bacteria. The conserved E5 and N-methylation might represent a  
471 bacterial solution to the problem of membrane escape for proteins anchored in the  
472 phospholipid membrane. The remarkable efficiency of this strategy and the dramatic effect of  
473 E5A mutation provide another example of the importance and the strength of weak bonds in  
474 biological systems.

475

476         Overall, the results of this study are compatible with a dual role of PulM binding to  
477 PulG. During initial steps of T2SS assembly, PulM might bind the PulG precursor to favour  
478 recruitment of pseudopilin-PulF subcomplex to the secretin/AP complex<sup>25</sup>. During fibre  
479 elongation, PulM might facilitate PulG targeting to the active site of the T2SS and/or its

480 extraction from the membrane, by favouring a conformation that minimises membrane  
481 contacts. These roles for PulM are based on its ability to bind PulG and PulH on one hand and  
482 PulL on the other, probably in a dynamic fashion, as shown for its homologues in T2SSs<sup>51</sup>  
483 and T4P<sup>52</sup>. Eventually, PulG recruited from the IM pool would reach the assembly focus  
484 defined by PulF and the PulE ATPase poised for membrane extraction and incorporation into  
485 the pseudopilus. Future studies are needed to reveal the nature of these conformational  
486 changes and the precise molecular function of PulM in coordinating PulG membrane  
487 extraction with its incorporation into the growing fibre.

488

## 489 **Materials and Methods**

490

### 491 *Bacterial strains and culture*

492

493 The *E. coli* DH5 $\alpha$  F' *lacI*<sup>Q</sup> strain was used for cloning purposes. Strain PAP7460  
494 [ $\Delta$ (*lac-argF*)U169 *araD139 relA1 rpsL150*  $\Delta$ *malE444 malG501* [F' (*lacI*<sup>Q</sup>  $\Delta$ *lacZM15 pro+*  
495 *Tn10*)] (*Tc*<sup>R</sup>)<sup>24</sup> was used for *pul* gene expression and strain PAP5299 [*araD139*  $\Delta$ (*argF-*  
496 *lac*)U169 *rpsL150 relA1 flb5301 deoC1ptsF25 thi pcnB::Tn10* (F' *lacI*<sup>Q</sup>)]<sup>33</sup> was employed  
497 for secretion assays. Bacteria were grown at 30 °C in LB medium (10 g L<sup>-1</sup> bacto tryptone; 5 g  
498 L<sup>-1</sup> yeast extract; 10 g L<sup>-1</sup> NaCl; pH 7.0) containing antibiotics as required: ampicillin, 100  $\mu$ g  
499 ml<sup>-1</sup>; chloramphenicol, 25  $\mu$ g ml<sup>-1</sup>; kanamycin, 20  $\mu$ g ml<sup>-1</sup>. Expression of *pul* genes cloned  
500 under the control of the *lac* promoter was induced with 1 mM isopropyl- $\beta$ -D-thiogalactoside  
501 (IPTG). Expression of *pul* genes was induced with 0.4% maltose in LB medium buffered with  
502 1/10 volume of M63B1 salt solution (modified from<sup>53</sup>: 13.6 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>; 2 g L<sup>-1</sup>  
503 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.2 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.5 mg L<sup>-1</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O; 1 mg L<sup>-1</sup> vitamin B1; pH 7.0).

504

505 *Recombinant DNA and plasmid construction*

506 The list of plasmids used in this study is shown in Table 1.

507 **Table 1.** Plasmids used in this study.

508

Name	Ori / Resistance <sup>a</sup>	Relevant characteristics	Reference
pCHAP8185	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes	35
pCHAP8184	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes $\Delta$ <i>pulG</i>	33
pCHAP8400	ColE1 / Ap <sup>R</sup>	All <i>pul</i> genes $\Delta$ <i>pulG</i> $\Delta$ <i>pulNO</i>	This study
pCHAP8200	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes $\Delta$ <i>pulE</i>	This study
pCHAP8218	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes $\Delta$ <i>pull</i>	35
pCHAP1217	ColE1 / Ap <sup>R</sup>	All <i>pul</i> genes $\Delta$ <i>pull</i>	24
pCHAP8496	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes $\Delta$ <i>pulM</i>	This study
pCHAP8251	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes $\Delta$ <i>pull</i>	This study
pCHAP8811	ColE1 / Ap <sup>R</sup>	<i>pulM</i>	This study
pCHAP576	ColE1 / Kan <sup>R</sup>	<i>pulO</i>	54
pCHAP8258	p15A / Cm <sup>R</sup>	<i>pulL</i>	This study
pCHAP1353	p15A / Cm <sup>R</sup>	<i>pulM</i>	24
pCHAP8843	p15A / Cm <sup>R</sup>	<i>pulL-pulM</i>	This study
pCHAP1362	p15A / Cm <sup>R</sup>	<i>pulG-his<sub>6</sub></i>	55
pCHAP7785	p15A / Cm <sup>R</sup>	<i>pulG<sup>E5A</sup>-his<sub>6</sub></i>	This study
pCHAP8658	p15A / Cm <sup>R</sup>	<i>pulG</i>	34
pCHAP8732	p15A / Cm <sup>R</sup>	<i>pulG<sup>T2A</sup></i>	This study
pCHAP8663	p15A / Cm <sup>R</sup>	<i>pulG<sup>E5A</sup></i>	34
pCHAP8875	ColE1 / Ap <sup>R</sup>	<i>pulA<sub>sol</sub></i> all <i>pul</i> genes $\Delta$ <i>pulG</i> $\Delta$ <i>pulM</i>	This study
pCHAP8377	ColE1 / Ap <sup>R</sup>	<i>pulKLMNO</i>	This study
pCHAP8395	ColE1 / Ap <sup>R</sup>	<i>pulKLM</i>	This study
pSU19	p15A / Cm <sup>R</sup>	Empty vector	56
pBGS18	ColE1 / Km <sup>R</sup>	Empty vector	57
pUT18c	ColE1 / Ap <sup>R</sup>	<i>placZ-T18</i>	36
pKT25	p15A / Km <sup>R</sup>	<i>placZ-T25</i>	36
pCHAP8154	ColE1/Ap <sup>R</sup>	pUT18c <i>pulM</i>	25
pCHAP8155	p15A/Km <sup>R</sup>	pKT25 <i>pulM</i>	25
pCHAP7330	ColE1/Ap <sup>R</sup>	pUT18c <i>pulG</i>	34
pCHAP7332	p15A/Km <sup>R</sup>	pKT25 <i>pulG</i>	34
pCHAP8670	ColE1/Ap <sup>R</sup>	pUT18c <i>pulG (E5A)</i>	34
pCHAP8420	p15A/Km <sup>R</sup>	pKT25 <i>pulG (E5A)</i>	34
pCHAP8733	ColE1/Ap <sup>R</sup>	pUT18c <i>pulG (T2A)</i>	This study
pCHAP8734	p15A/Km <sup>R</sup>	pKT25 <i>pulG (T2A)</i>	This study
pCHAP8429	ColE1/Ap <sup>R</sup>	pUT18c <i>pulG (P22A)</i>	This study
pCHAP8482	p15A/Km <sup>R</sup>	pKT25 <i>pulG (P22A)</i>	This study

509 a. Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.

510 Plasmid pCHAP8200 was generated by replacing the 7430-bp EcoRI-Bsu36I fragment

511 of plasmid pCHAP8185 with the corresponding fragment of plasmid pCHAP1230 carrying

512 the *ΔpulE* allele<sup>24</sup>. Plasmid pCHAP8251 was generated by replacing the EcoRI-HindIII  
513 fragment of plasmid pCHAP8185 with the corresponding fragment from pCHAP1217  
514 plasmid carrying the nonpolar deletion of the *pull* gene. Plasmid pCHAP8496 carrying a  
515 complete in-frame deletion of *pulM* was generated as follows. Random insertions of the GPS5  
516 ( $Km^R$ ) cassette in the *pulM* gene were generated *in vitro* using the GPS<sup>®</sup>-LS kit (New England  
517 Biolabs). Two insertions of the GPS5 cassette, each marked with a single PmeI site mapping  
518 in positions 20 and 120 of *pulM* ORF, were combined in plasmid pCHAP1353 to give  
519 pCHAP8370. The *pulM::kan* allele was introduced into pUC18 derivative containing the  
520 *pulM* gene, pCHAP8510, to give pCHAP8512. This plasmid was digested with PmeI to create  
521 an in-frame deletion of *pulM*, giving pCHAP8513. The DraIII-NsiI fragment containing the  
522 *ΔpulM* allele was ligated to the DraIII-NsiI fragment of pCHAP8377 containing the distal half  
523 of the *pul* operon, which was obtained by self ligating the AelI fragment of pCHAP231. This  
524 yielded plasmid pCHAP8516. In the final step, the NotI-EcoRI A fragment of pCHAP8516  
525 was ligated with the large EcoRI-NotI fragment of plasmid pCHAP8185 to give pCHAP8496.  
526 Plasmid pCHAP8811 was constructed by sub-cloning the EcoRI-HindIII insert containing the  
527 *pulM* gene from plasmid pCHAP1353 into pUC18 vector digested with EcoRI and HindIII.  
528 Plasmid pCHAP8732 carrying *pulG(T2A)* allele was generated by Quick-change mutagenesis  
529 using primers pulGT2A-5 and pulGT2A-3 (Table S1). Primers PulG T2A-Kpn and PulG Eco-  
530 3 were used to amplify the *pulG(T2A)* allele from plasmid pCHAP8732 in KpnI and EcoRI  
531 digested BAC2H plasmids pUT18c and pKT25, to yield pCHAP8733 (pUT18c-pulG<sup>T2A</sup>) and  
532 pCHAP8734 (pKT25-pulG<sup>T2A</sup>). Plasmid pCHAP8258 was made by cloning the *pull* gene  
533 PCR-amplified with primers Pull-Eco 5 and Pull-Hind 3 in the EcoRI and HindIII sites of  
534 pSU18. Plasmid pCHAP8843 was generated by cloning the PCR amplified pCHAP8185  
535 fragment containing the *pull* and *pulM* genes in pSU18 EcoRI and HindIII sites amplified by  
536 PCR using Pull-Eco 5 and PulM 3 oligonucleotide primers. All PCR reactions were



537 performed using the high-fidelity Pwo polymerase (Roche). Plasmid pCHAP8732 was  
538 derived from pCHAP8658 by the Quick-change method of site-directed mutagenesis with  
539 primers PulG T2A-5 and PulG T2A-3 (Table S1). Plasmid pCHAP8875 was constructed by  
540 ligating the pCHAP8184 NotI-HindIII B fragment with the pCHAP8496 NotI-HindIII  
541 fragment A. Plasmid pCHAP8400 was made in several steps. First, plasmid pCHAP8377 was  
542 constructed by ligating the AclI fragment of pCHAP231 containing the distal half of the *pul*  
543 operon. The EcoNI fragment of pCHAP8377 containing *pulN* and *pulO* genes was deleted to  
544 give pCHAP8395. The EcoRI-NotI fragments from pCHAP8395 and pCHAP8184 were then  
545 combined to reconstitute the *pul* operons with deletions of *pulG*, *pulN* and *pulO*.

546 All plasmid constructs were verified by sequencing (GATC). The list of  
547 oligonucleotides is provided in Table S1.

548

549 *In vivo cross-linking* and PulM-PulG interaction analysis.

550

551 For the formaldehyde cross-linking, 40 OD<sub>600</sub> of bacterial cultures were centrifuged  
552 (3500 x g, 5 minutes, room temperature). Bacterial pellets were washed with 10 ml PBS and  
553 resuspended in PBS at 8 OD<sub>600</sub> ml<sup>-1</sup>. Following the cross-linking with 0.6% formaldehyde  
554 (Sigma-Aldrich) for 20 minutes at 30°C with vigorous shaking, the bacteria were pelleted for  
555 2 minutes at ~12000 x g and resuspended in 5 ml of 50 mM Tris-HCl (pH 8.0) for 10 minutes  
556 at room temperature. The quenched reactions were centrifuged at 8000 x g for 5 minutes and  
557 the pellets were resuspended in 2 ml of cold TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4)  
558 containing cOmplete™ ULTRA EDTA-free (Roche) protease inhibitor cocktail  
559 (concentration as indicated by the supplier). The cells incubated on ice for 10 minutes in the  
560 presence of 0.2 mg ml<sup>-1</sup> lysozyme and 0.02 mg ml<sup>-1</sup> DNase I, followed by sonication at 4°C  
561 using the Vibra-Cell Ultrasonic Processor 75186 (Sonics & Materials) at 30% amplitude with

562 1 sec ON / 1 sec OFF cycles for a total sonication time of 10 seconds. The total cell extracts  
563 were analysed by SDS-PAGE and immunodetection as described below.

564

565 *Piliation and secretion assays*

566

567 Functional assays to test piliation and secretion were performed as described in <sup>34</sup>. For  
568 the shearing assay, bacteria were grown overnight in LB agar plates and expression of *pul*  
569 genes was induced by maltose (and IPTG, when required). Bacteria were scraped off the  
570 plates and resuspended in LB at 1 OD<sub>600</sub> ml<sup>-1</sup>, and 1 ml of the suspensions was vortexed for 1  
571 minute to detach surface pili. Upon centrifugation of the samples at ~12000 x g for 5 minutes  
572 at 4°C, the pelleted bacteria were resuspended in SDS sample buffer, and the supernatants  
573 containing the sheared pili were submitted to TCA (trichloroacetic acid) precipitation. Briefly,  
574 the supernatants were centrifuged at ~12000 x g for another 10 minutes at 4°C to remove any  
575 remaining bacteria, and pili were precipitated in 10% trichloroacetic acid for 30 minutes on  
576 ice. The precipitates were pelleted by centrifugation at ~12000 x g for 30 minutes at 4°C,  
577 washed twice with cold (-20°C) acetone, air-dried and resuspended in SDS sample buffer.  
578 Equal amounts of cell and sheared fractions were analysed using SDS-PAGE and PulG  
579 immunodetection. For the secretion assay, bacteria producing a non-acylated, soluble variant  
580 of PulA were grown in LB medium buffered with 0.1 volume of M63 salts, supplemented  
581 with 1mM IPTG and 0.2% maltose for the induction of *pul* genes. After normalization of  
582 cultures to 2 OD<sub>600</sub> ml<sup>-1</sup>, 1 ml was centrifuged at ~12000 x g for 5 minutes at 4°C. The  
583 pelleted cells were resuspended in SDS sample buffer. The supernatants were centrifuged  
584 again under the same conditions, and a sample was taken from the topmost part of the tube  
585 and mixed with 2 x SDS sample buffer. The same OD equivalent amounts of cell and  
586 supernatant fractions were analysed by SDS-PAGE and immunodetection of PulA.

587

588 *SDS-PAGE and immunodetection*

589

590 Protein separation was performed by SDS-PAGE in Tris-tricine gels<sup>58</sup> containing  
591 10% acrylamide, using Appalex or BioRad vertical gel electrophoresis systems. Proteins were  
592 electro-transferred onto Amersham Hybond ECL nitrocellulose membranes (GE Healthcare)  
593 using the semi-dry method with a buffer containing 5.8 g L<sup>-1</sup> Tris base, 2.9 g L<sup>-1</sup> glycine, 20%  
594 ethanol, 0.026% SDS. Membranes were blocked with 5% skim milk in TBST (10mM Tris-  
595 HCl, 15mM NaCl, 0.05% Tween20, pH 7.5-7.6), probed for 1 h with specific antiserum  
596 (1:2000 anti-PulG, 1:2000 anti-PulA, 1:1000 anti-PulL, 1:500 anti-PulM, 1:2000 anti-LamB,  
597 1:10000 anti-RbsB), washed several times with TBST, incubated with secondary antibody  
598 (1:20000 or 1:40000 anti-rabbit, horseradish peroxidase-coupled) for 1 h, and washed  
599 extensively with TBST. Membranes were developed by enhanced chemiluminescence using  
600 Pierce ECL 2 (Thermo Scientific), Western Lightning Plus ECL (PerkinElmer) or  
601 SuperSignal West Femto (Thermo Scientific), and the signal was recorded using Typhoon  
602 FLA 9000 imager (GE Healthcare) or LAS 4000 imager (Fujifilm). ImageJ software<sup>59</sup> was  
603 used for densitometric analysis of bands.

604

605 *Bacterial two-hybrid assay*

606

607 Competent cells of strain DHT1<sup>37</sup> were co-transformed with pUT18C and pKT25  
608 derivatives and bacteria were grown for 48 h at 30°C on LB plates containing Ap and Km. Six  
609 colonies were picked at random and inoculated into 5 ml cultures in LB containing Km and  
610 Ap, grown overnight and inoculated the next day into fresh medium containing 1 mM IPTG.  
611 Bacteria were cultured to mid-log phase and  $\beta$ -galactosidase activity was measured as

612 described <sup>60</sup>. At least 2 independent experiments were performed with several randomly  
613 picked transformants. Bar graphs represent mean values and error bars indicate standard  
614 deviation. The non-parametric Kruskal-Wallis followed by Dunn's multiple comparison tests  
615 were used in statistical analysis using the Graphpad Prism 6 software.

616

#### 617 *Immunofluorescence microscopy*

618

619 IF labelling of pili was performed as described previously <sup>34</sup>. Bacteria grown for 16  
620 hours at 30°C on LB agar supplemented with 0.4% maltose were carefully resuspended in  
621 PBS at 1 OD<sub>600</sub> ml<sup>-1</sup> and immobilized on coverslips coated with poly-L-lysine. After 30  
622 minutes of fixation using 3.7% formaldehyde at room temperature, reactions were quenched  
623 with 1M Tris-HCl pH 8.0 and samples were blocked with 1% bovine serum albumin (BSA) in  
624 PBS. PulG surface pili were detected using an anti-PulG antibody (1:1000) and a secondary  
625 anti-rabbit IgG coupled to Alexa Fluor 488 (1:200); bacteria were stained with 4',6-  
626 diamidino-2-phenylindole (DAPI). Samples were observed with an inverted Leica DMRIBe  
627 fluorescence microscope, and images were acquired with a Cool-Snap HQ CCD camera  
628 (Roper's Instruments). Pili number and length were quantified semi-automatically using the  
629 Metamorph software 6.1 (Universal Imaging) after having applied a shape filter to the images  
630 to omit round-shaped unspecific dots, which were present in all samples including the  
631 negative controls; the bacteria were counted manually using the same software. The non-  
632 specific labelling was not taken into consideration for the quantification. Note that since  
633 >95% of the fields in the  $\Delta pulM$  and  $\Delta pulM + pulG$  mutants did not have any pili, we defined  
634 a relevant field of vision as a field containing at least one pilus, and only relevant fields were  
635 taken into account for the quantification. The statistical significance of the differences was  
636 assessed by a Kruskal-Wallis test followed by a *post-hoc* analysis using the Dunn's multiple

637 comparison tests. Graphs corresponding to pili number and length generated were built using  
638 KaleidaGraph 4.1.

639

#### 640 *Molecular dynamics simulations*

641

642 A full-length model of PulG was derived from the X-ray crystal structure of the *K.*  
643 *oxytoca* PulG periplasmic domain (PDB ID: 1T92), as described in <sup>33</sup>. The missing 20  
644 carboxy-terminal residues were modelled on the basis of close homology to GspG from  
645 EHEC, and the TMS was modelled from PilA of *Pseudomonas aeruginosa* <sup>33</sup>. All residue  
646 mutations were performed using PyMOL (The PyMOL Molecular Graphics System, Version  
647 1.8 Schrödinger, LLC). The final PulG<sup>WT</sup> structure was composed of 133 amino acids and  
648 included one calcium cation; all ionisable groups were assigned to their most probable  
649 charged states at neutral pH.

650 PulG variants were then embedded in a pre-equilibrated palmitoyl-oleoyl  
651 phosphatidylethanolamine (POPE) membrane, using the GROMACS g\_membed tool <sup>61</sup>. PulG  
652 position along the z-axis was guided by the presence of non-polar and hydrophobic residues  
653 in the lower section of the  $\alpha$ -helix, and interfacial aromatic residue Trp94. The systems were  
654 solvated with TIP3P water, via superimposition of a pre-equilibrated box of waters, and a  
655 ~0.1 M concentration of NaCl, with dimensions ~90 x 90 x 162Å. The resulting systems  
656 contained ~ 36,500 water molecules and 314 lipids.

657 At each stage of system setup, steepest descent energy minimization was performed to  
658 relax the protein geometry and to remove steric clashes between protein/lipid/solvent. The  
659 system was equilibrated over 1.5 ns, during which position restraints, applied to all non-  
660 hydrogen protein atoms, were gradually removed to relax the protein structure, membrane and  
661 solvent. The Ca<sup>2+</sup> ion was unrestrained during the equilibration steps yet remained in the

662 original bound position. Finally, 200-350 ns production MD simulations were carried out. All  
663 simulations were performed using GROMACS<sup>62</sup> version 4.5<sup>63</sup>. The protein was treated using  
664 the CHARMM22/CMAP force field<sup>64</sup>, and POPE lipid molecules using the CHARMM36  
665 parameter set<sup>64</sup>. The parameters for MeF1 were formulated based on existing parameterized  
666 fragments. Equations of motion were integrated using the leapfrog method with a 2 fs time  
667 step, and the LINCS algorithm was used to constrain bond lengths<sup>65</sup>. Electrostatic  
668 interactions were computed using the Particle-Mesh-Ewald (PME) algorithm<sup>66</sup> and the real-  
669 space sum was cut off at 12Å. Van der Waals interactions were switched off between 10Å  
670 and 12Å. The neighbour list was updated every 10 steps. Simulations were performed using  
671 conditions of constant temperature (310 K) and pressure (1 atm) via the Bussi thermostat<sup>67</sup>,  
672 and semi-isotropic pressure-coupling using the Parrinello-Rahman barostat<sup>68</sup> with a coupling  
673 constant of 5 ps, under periodic-boundary conditions. Visual analyses and preparation of  
674 molecular graphics from the simulation trajectories were performed using VMD<sup>69</sup>. Further  
675 analysis was performed using GROMACS and graphs were prepared with Grace  
676 (<http://plasma-gate.weizmann.ac.il/Grace/>). Simulations were performed using the Darwin  
677 Supercomputer of the University of Cambridge High Performance Computing Service.

678

## 679 **Acknowledgements**

680

681 We thank Evelyne Richet and Jenny-Lee Thomassin for insightful comments and  
682 critical reading of the manuscript. We thank Cesar Valencia and Jenny-Lee Thomassin for  
683 help with IF data processing and statistical analysis, Mariette Bonnet for help and advice, and  
684 Stéphane Romero for stimulating discussions about biological fibres. We are grateful to all  
685 members of the Laboratory of Intercellular Communication and Microbial Infections and of  
686 the Laboratory for Macromolecular Systems and Signalling for helpful discussions and

687 friendly support. We thank Gouzel Karimova and Daniel Ladant for the strains, plasmids and  
688 advice concerning the BAC2H analysis. This work was funded by the ANR FiberSpace grant  
689 N°ANR-14-CE09-0004. Javier Santos-Moreno was funded by a fellowship from the Basque  
690 Government; Alexandra East was supported by an EPSRC PhD studentship. The  
691 computational work benefited from use of the Darwin Supercomputer of the University of  
692 Cambridge High Performance Computing Service (<http://www.hpc.cam.ac.uk/>), provided by  
693 Dell Inc. using Strategic Research, Infrastructure Funding from the Higher Education Funding  
694 Council for England. The authors state that they have no conflict of interest.

695  
696 **References**

- 697 1. Berry, J. L. & Pelicic, V. (2015). Exceptionally widespread nanomachines  
698 composed of type IV pilins: the prokaryotic Swiss Army knives. *FEMS Microbiology*  
699 *Reviews* 39, 134-54.
- 700 2. Jarrell, K. F. & Albers, S. V. (2012). The archaellum: an old motility structure with  
701 a new name. *Trends in Microbiology* 20, 307-12.
- 702 3. Makarova, K. S., Koonin, E. V. & Albers, S. V. (2016). Diversity and Evolution of  
703 Type IV pili Systems in Archaea. *Front Microbiol* 7, 667.
- 704 4. Pohlschroder, M. & Esquivel, R. N. (2015). Archaeal type IV pili and their  
705 involvement in biofilm formation. *Front Microbiol* 6, 190.
- 706 5. Melville, S. & Craig, L. (2013). Type IV pili in Gram-positive bacteria. *Microbiology*  
707 *and Molecular Biology Reviews* 77, 323-41.
- 708 6. Korotkov, K. V., Sandkvist, M. & Hol, W. G. (2012). The type II secretion system:  
709 biogenesis, molecular architecture and mechanism. *Nat Rev Microbiol* 10, 336-51.
- 710 7. Campos, M., Cisneros, D. A., Nivaskumar, M. & Francetic, O. (2013). The type II  
711 secretion system - a dynamic fiber assembly nanomachine. *Res Microbiol* 164, 545-55.
- 712 8. Nivaskumar, M. & Francetic, O. (2014). Type II secretion system: A magic  
713 beanstalk or a protein escalator. *Biochim Biophys Acta*.
- 714 9. Strom, M. S. & Lory, S. (1991). Amino acid substitutions in pilin of *Pseudomonas*  
715 *aeruginosa*. Effect on leader peptide cleavage, amino-terminal methylation, and pilus  
716 assembly. *J Biol Chem* 266, 1656-64.
- 717 10. Strom, M. S., Nunn, D. N. & Lory, S. (1993). A single bifunctional enzyme, PilD,  
718 catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family.

- 719 *Proceedings of the National Academy of Sciences of the United States of America* 90, 2404-  
720 8.
- 721 11. Strom, M. S., Bergman, P. & Lory, S. (1993). Identification of active-site cysteines  
722 in the conserved domain of PilD, the bifunctional type IV pilin leader peptidase/N-  
723 methyltransferase of *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* 268,  
724 15788-94.
- 725 12. Chang, Y. W., Rettberg, L. A., Treuner-Lange, A., Iwasa, J., Sogaard-Andersen, L. &  
726 Jensen, G. J. (2016). Architecture of the type IVa pilus machine. *Science* 351, aad2001.
- 727 13. Possot, O., d'Enfert, C., Reyss, I. & Pugsley, A. P. (1992). Pullulanase secretion in  
728 *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic  
729 cytoplasmic membrane protein. *Molecular Microbiology* 6, 95-105.
- 730 14. Py, B., Loiseau, L. & Barras, F. (2001). An inner membrane platform in the type II  
731 secretion machinery of Gram-negative bacteria. *EMBO Rep* 2, 244-8.
- 732 15. Chami, M., Guilvout, I., Gregorini, M., Remigy, H. W., Muller, S. A., Valerio, M., Engel,  
733 A., Pugsley, A. P. & Bayan, N. (2005). Structural insights into the secretin PulD and its  
734 trypsin-resistant core. *Journal of Biological Chemistry* 280, 37732-41.
- 735 16. Sauvonnet, N., Vignon, G., Pugsley, A. P. & Gounon, P. (2000). Pilus formation and  
736 protein secretion by the same machinery in *Escherichia coli*. *EMBO Journal* 19, 2221-8.
- 737 17. Durand, E., Bernadac, A., Ball, G., Lazdunski, A., Sturgis, J. N. & Filloux, A. (2003).  
738 Type II protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar  
739 and adhesive structure. *Journal of Bacteriology* 185, 2749-58.
- 740 18. Gray, M. D., Bagdasarian, M., Hol, W. G. & Sandkvist, M. (2011). In vivo cross-  
741 linking of EpsG to EpsL suggests a role for EpsL as an ATPase-pseudopilin coupling  
742 protein in the Type II secretion system of *Vibrio cholerae*. *Mol Microbiol* 79, 786-98.
- 743 19. Sandkvist, M., Hough, L. P., Bagdasarian, M. M. & Bagdasarian, M. (1999). Direct  
744 interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio*  
745 *cholerae*. *Journal of Bacteriology* 181, 3129-35.
- 746 20. Abendroth, J., Murphy, P., Sandkvist, M., Bagdasarian, M. & Hol, W. G. (2005). The  
747 X-ray structure of the type II secretion system complex formed by the N-terminal  
748 domain of EpsE and the cytoplasmic domain of EpsL of *Vibrio cholerae*. *J Mol Biol* 348,  
749 845-55.
- 750 21. Camberg, J. L., Johnson, T. L., Patrick, M., Abendroth, J., Hol, W. G. & Sandkvist, M.  
751 (2007). Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic  
752 phospholipids. *EMBO J* 26, 19-27.
- 753 22. Michel, G., Bleves, S., Ball, G., Lazdunski, A. & Filloux, A. (1998). Mutual  
754 stabilization of the XcpZ and XcpY components of the secretory apparatus in  
755 *Pseudomonas aeruginosa*. *Microbiology* 144 ( Pt 12), 3379-86.

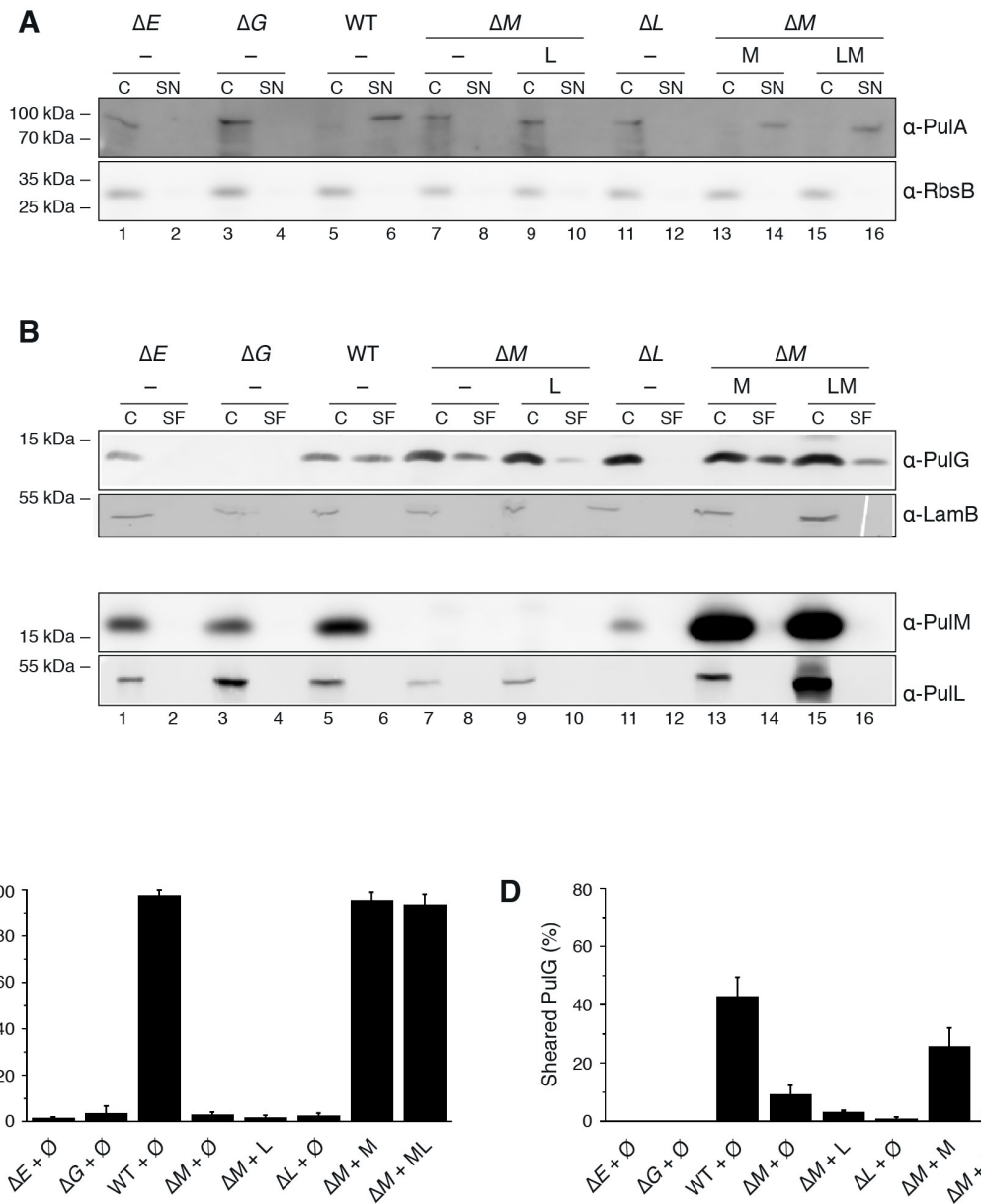


- 756 23. Johnson, T. L., Scott, M. E. & Sandkvist, M. (2007). Mapping critical interactive  
757 sites within the periplasmic domain of the *Vibrio cholerae* type II secretion protein  
758 EpsM. *Journal of Bacteriology* 189, 9082-9.
- 759 24. Possot, O. M., Vignon, G., Bomchil, N., Ebel, F. & Pugsley, A. P. (2000). Multiple  
760 interactions between pullulanase secretion components involved in stabilization and  
761 cytoplasmic membrane association of PulE. *J Bacteriol* 182, 2142-52.
- 762 25. Nivaskumar, M., Santos-Moreno, J., Malosse, C., Nadeau, N., Chamot-Rooke, J., Tran  
763 Van Nhieu, G. & Francetic, O. (2016). Pseudopilin residue E5 is essential for recruitment  
764 by the type 2 secretion system assembly platform. *Molecular Microbiology* 101, 924-41.
- 765 26. Georgiadou, M., Castagnini, M., Karimova, G., Ladant, D. & Pelicic, V. (2012). Large-  
766 scale study of the interactions between proteins involved in type IV pilus biology in  
767 *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly. *Mol*  
768 *Microbiol* 84, 857-73.
- 769 27. Karuppiah, V., Collins, R. F., Thistlethwaite, A., Gao, Y. & Derrick, J. P. (2013).  
770 Structure and assembly of an inner membrane platform for initiation of type IV pilus  
771 biogenesis. *Proc Natl Acad Sci U S A* 110, E4638-47.
- 772 28. Tammam, S., Sampaleanu, L. M., Koo, J., Manoharan, K., Daubaras, M., Burrows, L.  
773 L. & Howell, P. L. (2013). PilMNOPQ from the *Pseudomonas aeruginosa* type IV pilus  
774 system form a transenvelope protein interaction network that interacts with PilA. *J*  
775 *Bacteriol* 195, 2126-35.
- 776 29. Pasloske, B. L., Scraba, D. G. & Paranchych, W. (1989). Assembly of mutant pilins  
777 in *Pseudomonas aeruginosa*: formation of pili composed of heterologous subunits.  
778 *Journal of Bacteriology* 171, 2142-7.
- 779 30. Aas, F. E., Winther-Larsen, H. C., Wolfgang, M., Frye, S., Lovold, C., Roos, N., van  
780 Putten, J. P. & Koomey, M. (2007). Substitutions in the N-terminal alpha helical spine of  
781 *Neisseria gonorrhoeae* pilin affect Type IV pilus assembly, dynamics and associated  
782 functions. *Molecular Microbiology* 63, 69-85.
- 783 31. Pugsley, A. P. (1993). Processing and methylation of PulG, a pilin-like component  
784 of the general secretory pathway of *Klebsiella oxytoca*. *Mol Microbiol* 9, 295-308.
- 785 32. Vignon, G., Kohler, R., Larquet, E., Giroux, S., Prevost, M. C., Roux, P. & Pugsley, A.  
786 P. (2003). Type IV-like pili formed by the type II secretion: specificity, composition,  
787 bundling, polar localization, and surface presentation of peptides. *J Bacteriol* 185, 3416-  
788 28.
- 789 33. Campos, M., Nilges, M., Cisneros, D. A. & Francetic, O. (2010). Detailed structural  
790 and assembly model of the type II secretion pilus from sparse data. *Proc Natl Acad Sci U*  
791 *SA* 107, 13081-6.
- 792 34. Nivaskumar, M., Bouvier, G., Campos, M., Nadeau, N., Yu, X., Egelman, E. H., Nilges,  
793 M. & Francetic, O. (2014). Distinct docking and stabilization steps of the pseudopilus  
794 conformational transition path suggest rotational assembly of type IV pilus-like fibers.  
795 *Structure* 22, 685-96.

- 796 35. Cisneros, D. A., Bond, P. J., Pugsley, A. P., Campos, M. & Francetic, O. (2012). Minor  
797 pseudopilin self-assembly primes type II secretion pseudopilus elongation. *EMBO J* 31,  
798 1041-53.
- 799 36. Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. (1998). A bacterial two-hybrid  
800 system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A*  
801 95, 5752-6.
- 802 37. Dautin, N., Karimova, G., Ullmann, A. & Ladant, D. (2000). Sensitive genetic screen  
803 for protease activity based on a cyclic AMP signaling cascade in *Escherichia coli*. *Journal*  
804 *of Bacteriology* 182, 7060-6.
- 805 38. Durand, E., Michel, G., Voulhoux, R., Kurner, J., Bernadac, A. & Filloux, A. (2005).  
806 XcpX controls biogenesis of the *Pseudomonas aeruginosa* XcpT-containing pseudopilus. *J*  
807 *Biol Chem* 280, 31378-89.
- 808 39. Lybarger, S. R., Johnson, T. L., Gray, M. D., Sikora, A. E. & Sandkvist, M. (2009).  
809 Docking and assembly of the type II secretion complex of *Vibrio cholerae*. *Journal of*  
810 *Bacteriology* 191, 3149-61.
- 811 40. Buddelmeijer, N., Krehenbrink, M., Pecorari, F. & Pugsley, A. P. (2009). Type II  
812 secretion system secretin PulD localizes in clusters in the *Escherichia coli* outer  
813 membrane. *Journal of Bacteriology* 191, 161-8.
- 814 41. Kolappan, S., Coureuil, M., Yu, X., Nassif, X., Egelman, E. H. & Craig, L. (2016).  
815 Structure of the *Neisseria meningitidis* Type IV pilus. *Nat Commun* 7, 13015.
- 816 42. Craig, L., Volkmann, N., Arvai, A. S., Pique, M. E., Yeager, M., Egelman, E. H. &  
817 Tainer, J. A. (2006). Type IV pilus structure by cryo-electron microscopy and  
818 crystallography: implications for pilus assembly and functions. *Mol Cell* 23, 651-62.
- 819 43. Craig, L., Taylor, R. K., Pique, M. E., Adair, B. D., Arvai, A. S., Singh, M., Lloyd, S. J.,  
820 Shin, D. S., Getzoff, E. D., Yeager, M., Forest, K. T. & Tainer, J. A. (2003). Type IV pilin  
821 structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated  
822 pilus and *Pseudomonas aeruginosa* PAK pilin. *Molecular Cell* 11, 1139-50.
- 823 44. Lemkul, J. A. & Bevan, D. R. (2011). Characterization of interactions between PilA  
824 from *Pseudomonas aeruginosa* strain K and a model membrane. *J Phys Chem B* 115,  
825 8004-8.
- 826 45. Andersson, H. & von Heijne, G. (1994). Membrane protein topology: effects of  
827  $\Delta\mu H^+$  on the translocation of charged residues explain the 'positive inside' rule.  
828 *EMBO Journal* 13, 2267-72.
- 829 46. Pasloske, B. L. & Paranchych, W. (1988). The expression of mutant pilins in  
830 *Pseudomonas aeruginosa*: fifth position glutamate affects pilin methylation. *Mol*  
831 *Microbiol* 2, 489-95.
- 832 47. Thomas, N. A., Bardy, S. L. & Jarrell, K. F. (2001). The archaeal flagellum: a  
833 different kind of prokaryotic motility structure. *FEMS Microbiology Reviews* 25, 147-74.

- 834 48. Albers, S. V. & Meyer, B. H. (2011). The archaeal cell envelope. *Nat Rev Microbiol*  
835 9, 414-26.
- 836 49. Ota, A., Gmajner, D., Sentjurc, M. & Ulrih, N. P. (2012). Effect of growth medium pH  
837 of *Aeropyrum pernix* on structural properties and fluidity of archaeosomes. *Archaea*  
838 2012, 285152.
- 839 50. Trincone, A., Nicolaus, B., Palmieri, G., De Rosa, M., Huber, R., Huber, G., Stetter, K.  
840 O. & Gambacorta, A. (1992). Distribution of complex and core lipids within new  
841 hyperthermophilic members of the *Archaea* domain. *Systematic and Applied*  
842 *Microbiology* 15, 11-17.
- 843 51. Lallemand, M., Login, F. H., Guschinskaya, N., Pineau, C., Effantin, G., Robert, X. &  
844 Shevchik, V. E. (2013). Dynamic interplay between the periplasmic and transmembrane  
845 domains of GspL and GspM in the type II secretion system. *PLoS One* 8, e79562.
- 846 52. Leighton, T. L., Dayalani, N., Sampaleanu, L. M., Howell, P. L. & Burrows, L. L.  
847 (2015). Novel Role for PilNO in Type IV Pilus Retraction Revealed by Alignment  
848 Subcomplex Mutations. *Journal of Bacteriology* 197, 2229-38.
- 849 53. Pardee, A. B., Jacob, F. & Monod, J. (1959). The genetic control and cytoplasmic  
850 expression of "inducibility" in the synthesis of  $\beta$ -galactosidase by *E. coli*. *J Mol Biol* 1,  
851 165-178.
- 852 54. Pugsley, A. P., Bayan, N. & Sauvonnet, N. (2001). Disulfide bond formation in  
853 secretion component PulK provides a possible explanation for the role of DsbA in  
854 pullulanase secretion. *Journal of Bacteriology* 183, 1312-9.
- 855 55. Kohler, R., Schafer, K., Muller, S., Vignon, G., Diederichs, K., Philippsen, A., Ringler,  
856 P., Pugsley, A. P., Engel, A. & Welte, W. (2004). Structure and assembly of the pseudopilin  
857 PulG. *Mol Microbiol* 54, 647-64.
- 858 56. Bartolome, B., Jubete, Y., Martinez, E. & de la Cruz, F. (1991). Construction and  
859 properties of a family of pACYC184-derived cloning vectors compatible with pBR322  
860 and its derivatives. *Gene* 102, 75-8.
- 861 57. Spratt, B. G., Hedge, P. J., te Heesen, S., Edelman, A. & Broome-Smith, J. K. (1986).  
862 Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and  
863 pEMBL9. *Gene* 41, 337-42.
- 864 58. Schagger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-  
865 polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to  
866 100 kDa. *Analytical Biochemistry* 166, 368-79.
- 867 59. Abràmoff, M. D., Magalhães, P. J. & Ram, S. J. (2004). Image processing with  
868 ImageJ. *Biophoton Int* 11, 36-43.
- 869 60. Miller, J. H. (1972). *Experiments in molecular genetics*, Cold Spring Harbor  
870 Laboratory, Cold Spring Harbor, New York.

- 871 61. Wolf, M. G., Hoefling, M., Aponte-Santamaria, C., Grubmuller, H. & Groenhof, G.  
872 (2010). g\_membed: Efficient insertion of a membrane protein into an equilibrated lipid  
873 bilayer with minimal perturbation. *J Comput Chem* 31, 2169-74.
- 874 62. Hess, B., Kutzner, C., van der Spoel, D. & Lindahl, E. (2008). GROMACS 4:  
875 Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. *J*  
876 *Chem Theory Comput* 4, 435-47.
- 877 63. Bjelkmar, P., Larsson, P., Cuendet, M. A., Hess, B. & Lindahl, E. (2010).  
878 Implementation of the CHARMM Force Field in GROMACS: Analysis of Protein Stability  
879 Effects from Correction Maps, Virtual Interaction Sites, and Water Models. *J Chem Theory*  
880 *Comput* 6, 459-66.
- 881 64. MacKerell, A. D., Bashford, D., Bellott, M., Dunbrack, R. L., Evanseck, J. D., Field, M.  
882 J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczera, K., Lau, F.  
883 T., Mattos, C., Michnick, S., Ngo, T., Nguyen, D. T., Prodhom, B., Reiher, W. E., Roux, B.,  
884 Schlenkrich, M., Smith, J. C., Stote, R., Straub, J., Watanabe, M., Wiorkiewicz-Kuczera, J.,  
885 Yin, D. & Karplus, M. (1998). All-atom empirical potential for molecular modeling and  
886 dynamics studies of proteins. *J Phys Chem B* 102, 3586-616.
- 887 65. Hess, B. (1997). Periodic patterns in biochemical reactions. *Quarterly Reviews of*  
888 *Biophysics* 30, 121-76.
- 889 66. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H. & Pedersen, L. G.  
890 (1995). A smooth particle mesh Ewald potential. *Journal of Chemical Physics* 103, 8577-  
891 8592.
- 892 67. Bussi, G., Donadio, D. & Parrinello, M. (2007). Canonical sampling through  
893 velocity rescaling. *Journal of Chemical Physics* 126, 014101.
- 894 68. Parrinello, M. & Rahman, A. (1981). Polymorphic transitions in single crystals: A  
895 new molecular dynamics method. *J Appl Phys* 52, 7182-7190.
- 896 69. Humphrey, W., Dalke, A. & Schulten, K. (1996). VMD: visual molecular dynamics.  
897 *Journal of Molecular Graphics* 14, 33-8, 27-8.
- 898 70. Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. & Barton, G. J. (2009).  
899 Jalview Version 2-a multiple sequence alignment editor and analysis workbench.  
900 *Bioinformatics* 25, 1189-91.  
901  
902  
903



905

906 **Fig. 1. The role of PulM in T2SS function**

907 A. PulA secretion assay in near-chromosomal expression conditions using *E. coli* PAP5299  
 908 co-transformed with pCHAP8185 (all *pul* genes, WT) or its derivatives containing single  
 909 nonpolar *pul* gene deletions as indicated by a single letter code:  $\Delta E$  (pCHAP8200),  $\Delta G$   
 910 (pCHAP8184),  $\Delta M$  (pCHAP8496), or  $\Delta L$  (pCHAP8251); and with compatible pSU19 (-) or  
 911 its derivatives carrying indicated *pul* genes: L (pCHAP8258), M (pCHAP1353) or LM  
 912 (pCHAP8843). The amount of pullulanase PulA in 0.015 OD<sub>600</sub> units of cell extracts (C) and

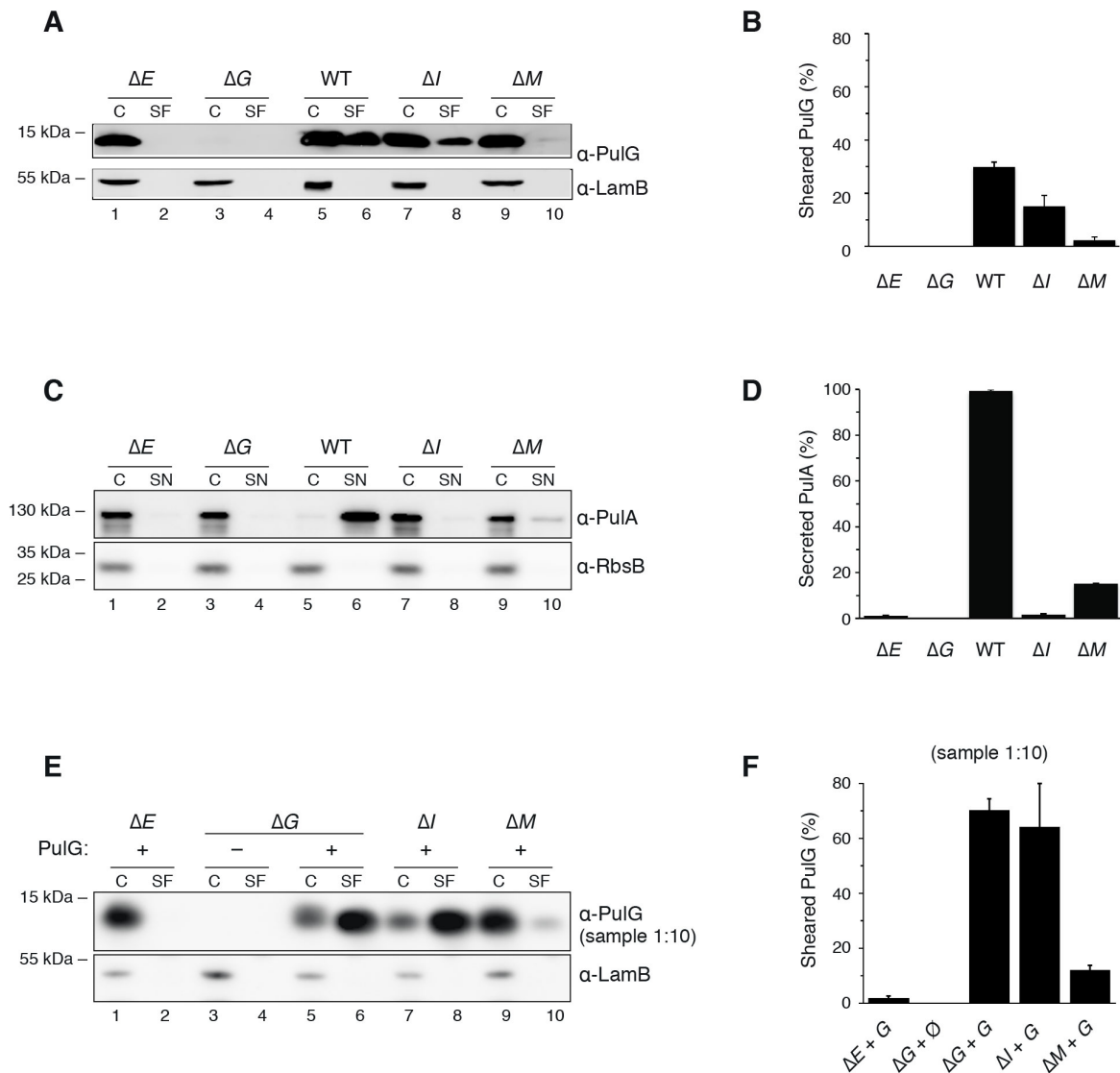
913 culture supernatants (SN) was assessed by Western blot. Immunodetection of the periplasmic  
914 ribose-binding protein RbsB in 0.03 OD<sub>600</sub> units served as a lysis control. Molecular weight  
915 (Mw) markers and lane numbers are shown.

916 B. PulG pilus assembly assay of *E. coli* PAP7460 overexpressing the *pul* genes from the same  
917 plasmids as in (A). Cell (C) and sheared pili (SF) fractions from an equivalent of 0.05 OD<sub>600</sub>  
918 units were separated on Tris-Tricin SDS-PAGE, transferred onto nitrocellulose membranes  
919 and probed with antibodies against PulG, LamB, PulM and Pull. Mw markers and lane  
920 numbers are shown.

921 C. Quantification of the percentage of secreted Pula (mean + standard deviation - s.d.) from  
922 three independent experiments as the one in (A). Ø indicates empty vector.

923 D. Quantification of the percentage of sheared PulG (mean + s.d.) from three independent  
924 experiments like the one in (B). Ø, empty vector.

925



926

927 **Fig. 2. Characterisation of  $\Delta pulM$  mutant function under conditions of *pul* gene**  
 928 **overexpression.**

929 Shearing (A and E) and secretion (C) assays and the corresponding quantifications of the  
 930 percentage of sheared PulG (mean + s.d.) (B and F) or secreted PulA (D) in three independent  
 931 experiments.

932 A. Immunodetection of PulG 0.05 OD<sub>600</sub> units of cell (C) and sheared fractions (SF) of *E. coli*  
 933 PAP7460 transformed with plasmid pCHAP8185 containing all *pul* genes (WT) or its single  
 934 gene deletion derivatives:  $\Delta E$  (pCHAP8200),  $\Delta G$  (pCHAP8184),  $\Delta I$  (pCHAP8218) and  $\Delta M$   
 935 (pCHAP8496). LamB immuno-detection is shown as control. Mw markers are indicated on  
 936 the left.

937 B. Percent of sheared PulG (mean + s.d.) from three independent experiments like the one  
938 shown in (A).

939 C. PulA secretion assay using transformed PAP7460 derivatives as in (A). PulA was detected  
940 by Western blot in 0.02 OD<sub>600</sub> units of cell extracts (C) and culture supernatants (SN).  
941 Immunodetection of periplasmic RbsB served as a lysis control. Mw markers and lane  
942 numbers are shown.

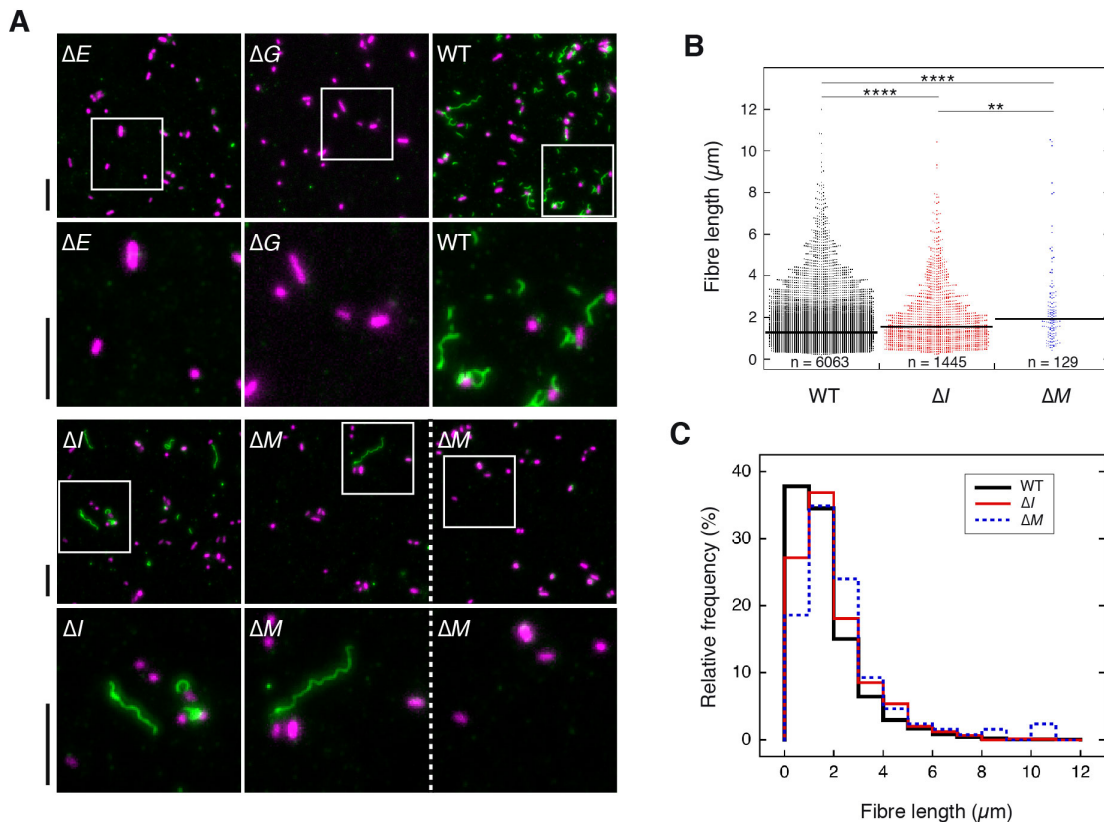
943 D. Quantification of the percentage of secreted PulA (mean + s.d.) from three independent  
944 experiments like the one in (C).

945 E. Effect of *pulG* overexpression on pseudopilus assembly. Top: Anti-PulG immunoblot of  
946 0.005 OD<sub>600</sub> units (“sample 1:10”) of cell (C) and sheared fractions (SF) of bacteria  
947 transformed with pSU19 (-) or its derivative pCHAP8658 encoding PulG (+) and indicated  
948 *pul* gene expression plasmids containing single gene deletions, as in (A):  $\Delta E$  (pCHAP8200),  
949  $\Delta G$  (pCHAP8184),  $\Delta I$  (pCHAP8218), or  $\Delta M$  (pCHAP8496). Bottom: Anti-LamB control of a  
950 10-fold concentrated sample (i.e. 0.05 OD<sub>600</sub> units). The position of the Mw markers is  
951 indicated.

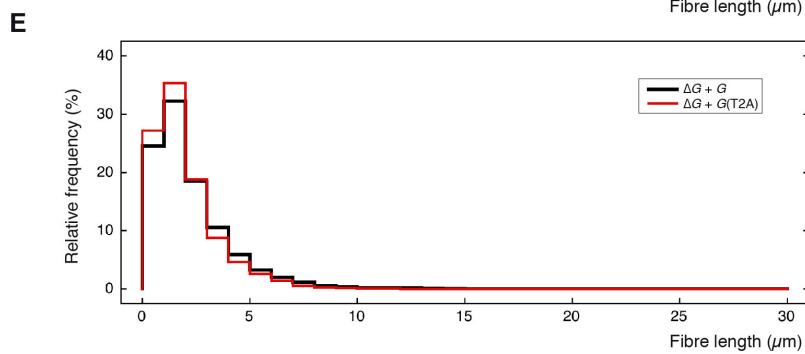
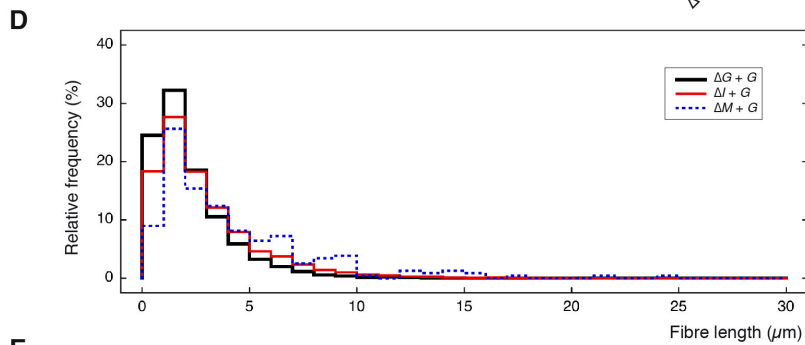
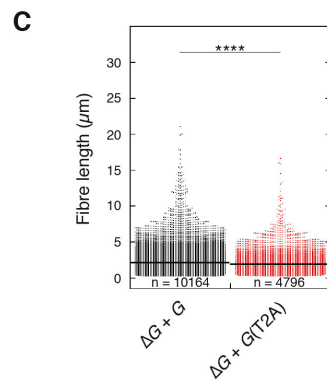
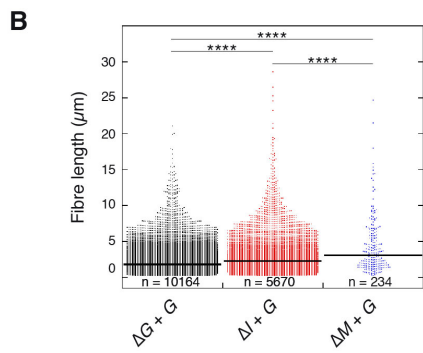
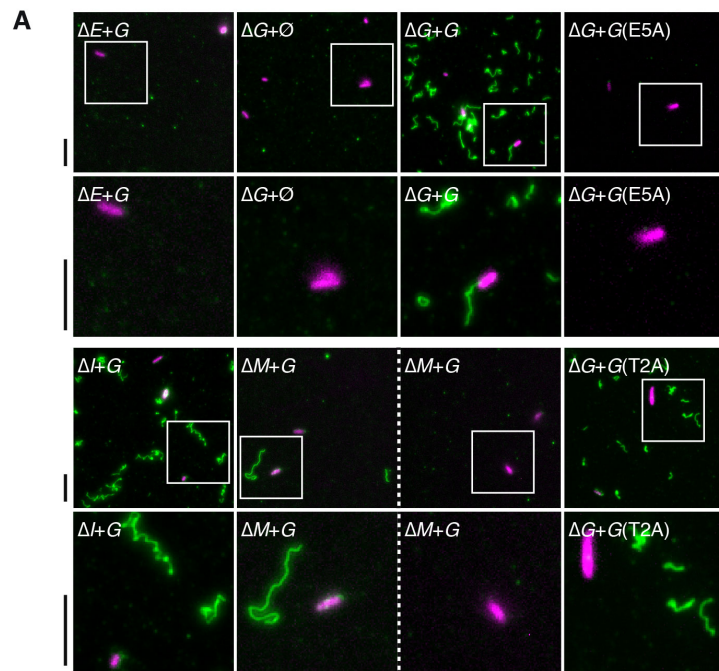
952 F. Percent (mean + s.d.) of PulG in the sheared fraction from three independent experiments  
953 as in (E), using 0.005 OD<sub>600</sub> units (“sample 1:10”).  $\emptyset$ , empty vector.

954





955  
 956 **Fig. 3. Quantitative IF microscopy analysis of piliation in  $\Delta pulM$  mutants.**  
 957 Bacteria (magenta) and PulG fibres (green) were labelled and examined by IF (Materials and  
 958 Methods).  
 959 A. Representative fluorescence micrographs. WT: wild-type;  $\Delta E$ :  $\Delta pulE$ ;  $\Delta G$ :  $\Delta pulG$ ;  $\Delta I$ :  
 960  $\Delta pullI$ ;  $\Delta M$ :  $\Delta pulM$ . A 2.6-fold magnified inset is shown below each image. An additional  
 961 panel is shown for  $\Delta pulM$  to illustrate the absence of fibres in the majority of the fields. Scale  
 962 bars = 5  $\mu\text{m}$ .  
 963 B. Dot-plot representing the length of individual fibres (dots) scored for 12864, 13062 and  
 964 14786 bacteria (for WT,  $\Delta pullI$  and  $\Delta pulM$  respectively) in three independent experiments. On  
 965 average,  $0.47 \pm 0.09$  (mean  $\pm$  s.d.,) fibres per bacterium were found in WT,  $0.11 \pm 0.2$  in  
 966  $\Delta pullI$  and  $0.009 \pm 0.003$  in  $\Delta pulM$  strain). The median fibre length values, shown as  
 967 horizontal black lines, are  $1.2 \pm 0.6 \mu\text{m}$  (median  $\pm$  m.a.d., median absolute deviation) for WT,  
 968  $1.5 \pm 0.7 \mu\text{m}$  for  $\Delta pullI$  strain and  $1.8 \pm 0.7 \mu\text{m}$  for  $\Delta pulM$  strain. Dunn's test,  $**p < 0.01$ ,  
 969  $****p < 0.0001$ .  
 970 C. Relative frequency distribution of fibre length. Bin size = 1  $\mu\text{m}$ .

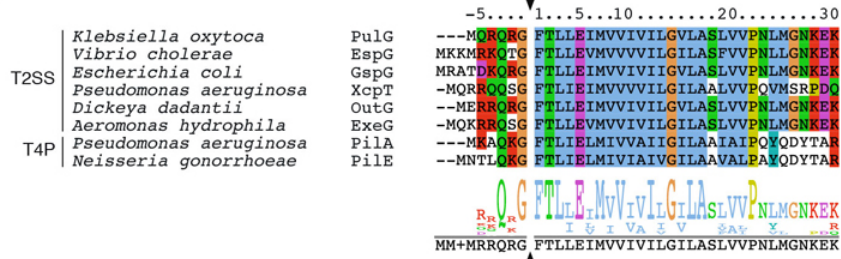
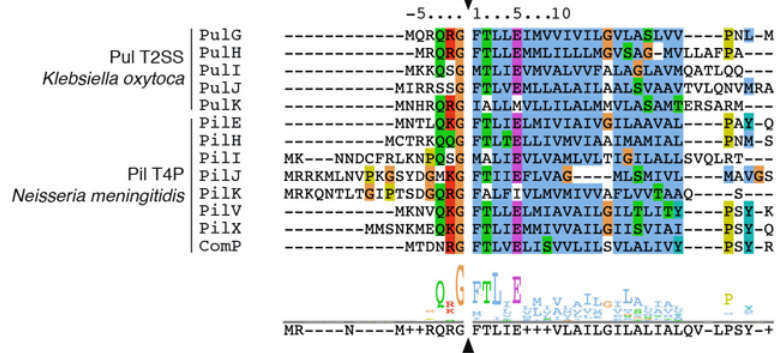
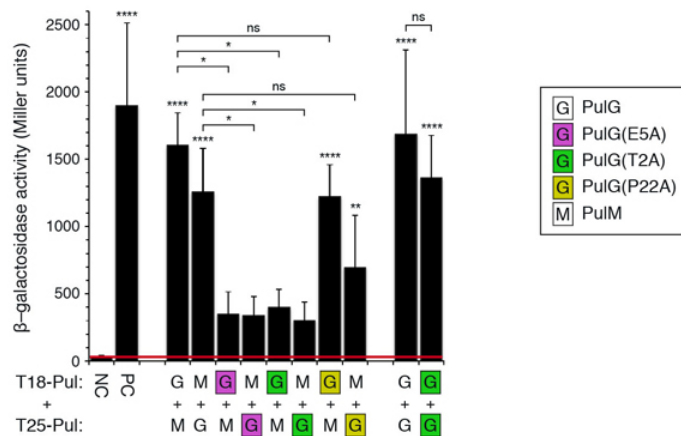
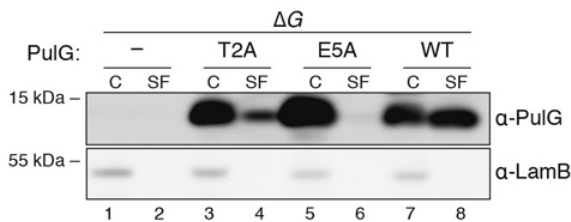
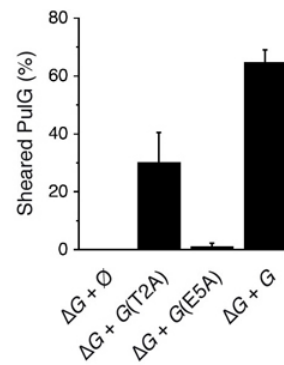
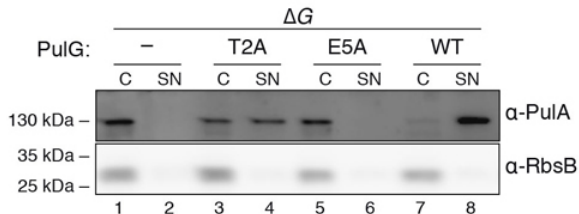
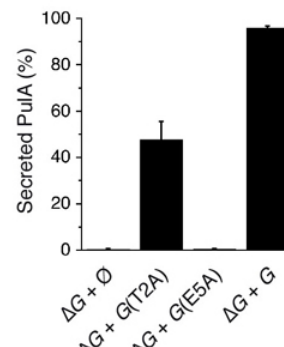


972 **Fig. 4. The effect of PulG overproduction on T2S pilus assembly.**

973 A. IF microscopy of the piliation ability of  $\Delta pulM + pulG$  and  $\Delta pulG + pulG(T2A)$ . Bacteria,  
974 magenta; fibres, green. A 2.6-fold magnified inset is shown below each image. An extra panel  
975 is shown for  $\Delta pulM + pulG$  to illustrate the absence of fibres in most of the fields. The scale  
976 bars (5  $\mu\text{m}$ ) are shown on the left.

977 B and C. Dot-plots representing the length of individual fibres (dots) scored for 1342, 1190,  
978 1597 and 1460 bacteria (for  $\Delta pulG + pulG$ ,  $\Delta pull + pulG$ ,  $\Delta pulM + pulG$  and  $\Delta pulG +$   
979  $pulG(T2A)$ , respectively) in three independent experiments. The number of fibres/bacterium  
980 (expressed as mean  $\pm$  s.d) were  $7.64 \pm 1.49$  (for strain  $\Delta pulG + pulG$ );  $4.7 \pm 0.6$  (for strain  
981  $\Delta pull + pulG$ );  $0.15 \pm 0.06$  (for strain  $\Delta pulM + pulG$ ); and  $3.73 \pm 1.68$  (for strain  $\Delta pulG +$   
982  $pulG(T2A)$ ). The median values of fibre length are shown as a horizontal black lines: for  
983  $\Delta pulG + pulG$ ,  $1.7 \pm 0.8 \mu\text{m}$  (median  $\pm$  m.a.d.); for  $\Delta pull + pulG$ ,  $2.2 \pm 1.1 \mu\text{m}$ ; for  $\Delta pulM +$   
984  $pulG$ ,  $3.0 \pm 1.6 \mu\text{m}$  and for  $\Delta pulG + pulG(T2A)$ ,  $1.6 \pm 0.7 \mu\text{m}$ . Dunn's test, \*\*\*\*p < 0.0001.

985 D and E. Relative frequency distributions of fibre length. Bin = 1  $\mu\text{m}$ .

**A****B****C****D****E****F****G**

987 **Fig. 5. PulG<sup>T2A</sup> variant shows defective interaction with PulM, piliation and secretion.**

988 A. Protein sequence alignment of the N-terminal segments of major T2SS pseudopilin and  
989 T4P subunits from the indicated bacterial species. The consensus sequence is indicated below  
990 and arrowheads indicate the prepilin peptidase cleavage site.

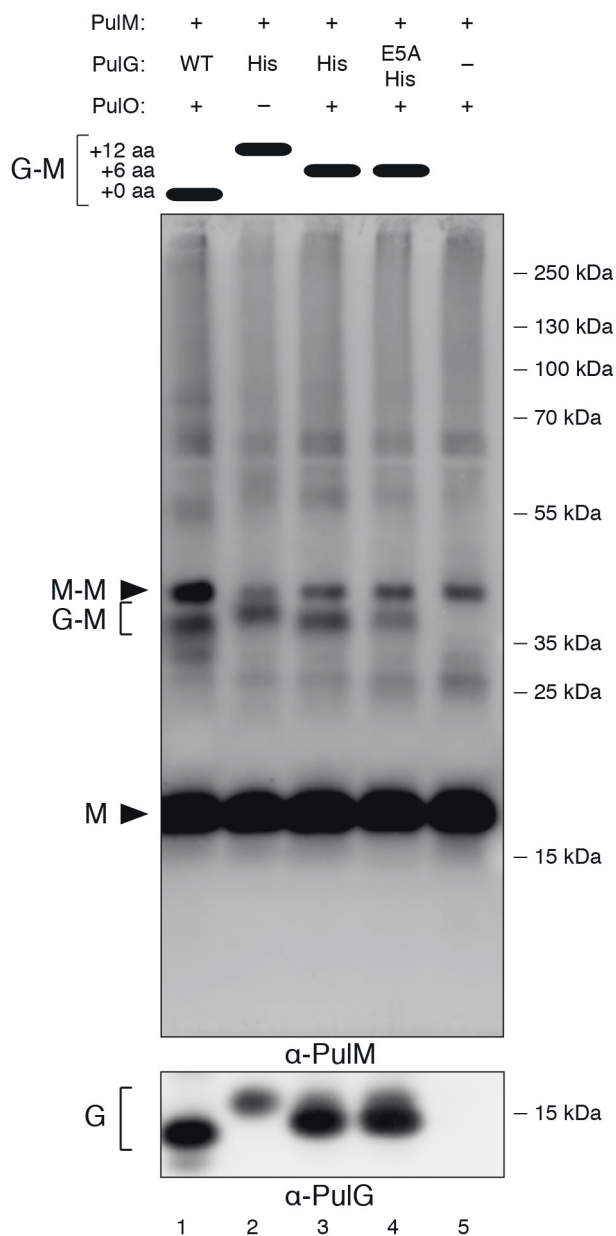
991 B. Sequence alignment of major and minor pseudopilins from the Pul T2SS of *Klebsiella*  
992 *oxytoca* (top) and major and minor T4P pilins from *Neisseria meningitidis* (bottom). The  
993 arrowheads indicate the prepilin peptidase cleavage site. Alignments were generated using  
994 Jalview2 software<sup>70</sup>.

995 C. BAC2H analysis of the interactions between T18 and T25 hybrids with PulM and PulG  
996 and its derivatives PulG<sup>E5A</sup>, PulG<sup>T2A</sup> and PulG<sup>P22A</sup>.  $\beta$ -galactosidase activity was measured in at  
997 least 6 independent colonies co-transformed with pUT18c and pKT25 plasmid derivatives  
998 (Table 1) as described in Materials and Methods. Bar graphs indicate mean values and error  
999 bars indicate standard deviation. Statistically significant values relative to the negative control  
1000 are indicated above each bar. The difference between certain positive interactions was  
1001 assessed for statistical significance and represented by horizontal lines indicating the  
1002 compared strains. NC, activity of bacteria producing T18 and T25 as negative control; PC,  
1003 activity of positive control strain producing T18-Zip and T25-Zip chimera. The red horizontal  
1004 line indicates the background mean  $\beta$ -galactosidase activity measured in the negative control.  
1005 \*p < 0.05; \*\*p < 0.01; \*\*\*\*p < 0.0001; ns, non-significant.

1006 D. Shearing assay of *E. coli* PAP7460 co-transformed with plasmid pCHAP8184 containing  
1007 all *pul* genes except *pulG* ( $\Delta G$ ) complemented with pSU19 (-) or its derivatives carrying *pulG*  
1008 alleles: WT (pCHAP8658), T2A (pCHAP8732) or E5A (pCHAP8663). PulG  
1009 immunodetection in 0.05 OD<sub>600</sub> units of cell and sheared fractions (C, SF). Below,  
1010 immunodetection of LamB in the same samples. Lane numbers and Mw markers are depicted.  
1011 E. Quantification of the percentage of sheared PulG (mean + s.d.) from three independent  
1012 experiments like the one in (D).  $\emptyset$  indicates an empty vector.

1013 F. Secretion assay using *E. coli* PAP5299 transformed with the same plasmids as in (D).  
1014 Immunodetection of PulA in 0.02 OD<sub>600</sub> units of cell extracts (C) and supernatants (SN) is  
1015 shown.  $\alpha$ -RbsB is used as a lysis control. Mw markers and lane numbers are shown.

1016 G. Quantification of the percentage of secreted PulA (mean + s.d.) from three independent  
1017 assays like the one in (F).  $\emptyset$  indicates empty vector.

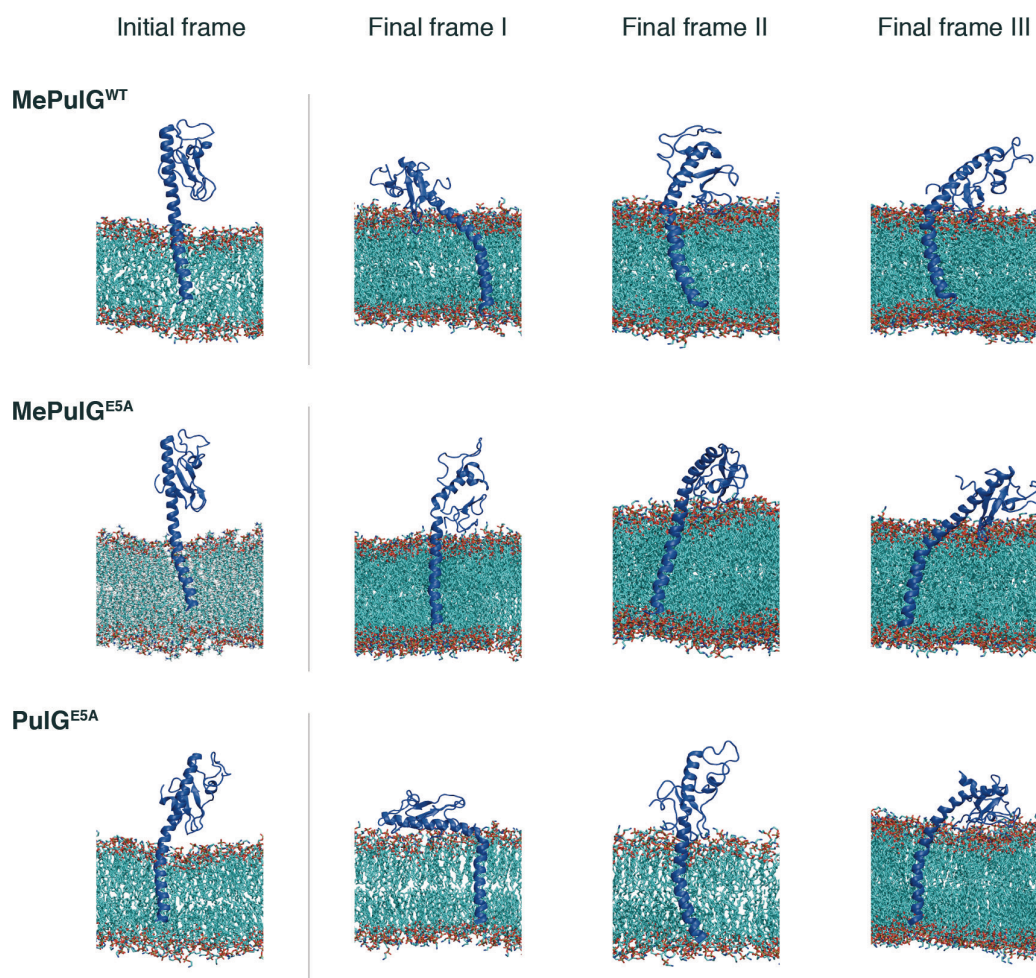


1018

1019 **Fig. 6. PulM interacts with PulG in the context of the complete Pul T2SS.**

1020 *E. coli* PAP7460 carrying plasmids encoding the *pul* genes with either  $\Delta pulG$  (pCHAP8184,  
 1021 lanes 1 and 3-5) or  $\Delta pulG + \Delta pulNO$  (pCHAP8400, lane 2) alleles were transformed with  
 1022 pSU19 or its derivatives encoding PulG (pCHAP8658), PulG-His<sub>6</sub> (pCHAP1362) or PulG<sup>E5A</sup>-  
 1023 His<sub>6</sub> (pCHAP7785). Total extracts of strains cross-linked *in vivo* with 0.6% FA (Materials and  
 1024 Methods) analysed by SDS-PAGE and immunodetection with anti-PulM or anti-PulG antisera  
 1025 are shown. Theoretical relative migration of PulM-PulG heterodimers is depicted according to  
 1026 the molecular weight of the PulG variant in each case. The positions of the PulM monomers  
 1027 (M), PulM homodimers (M-M) and the shifting PulG-PulM heterodimers (G-M) are

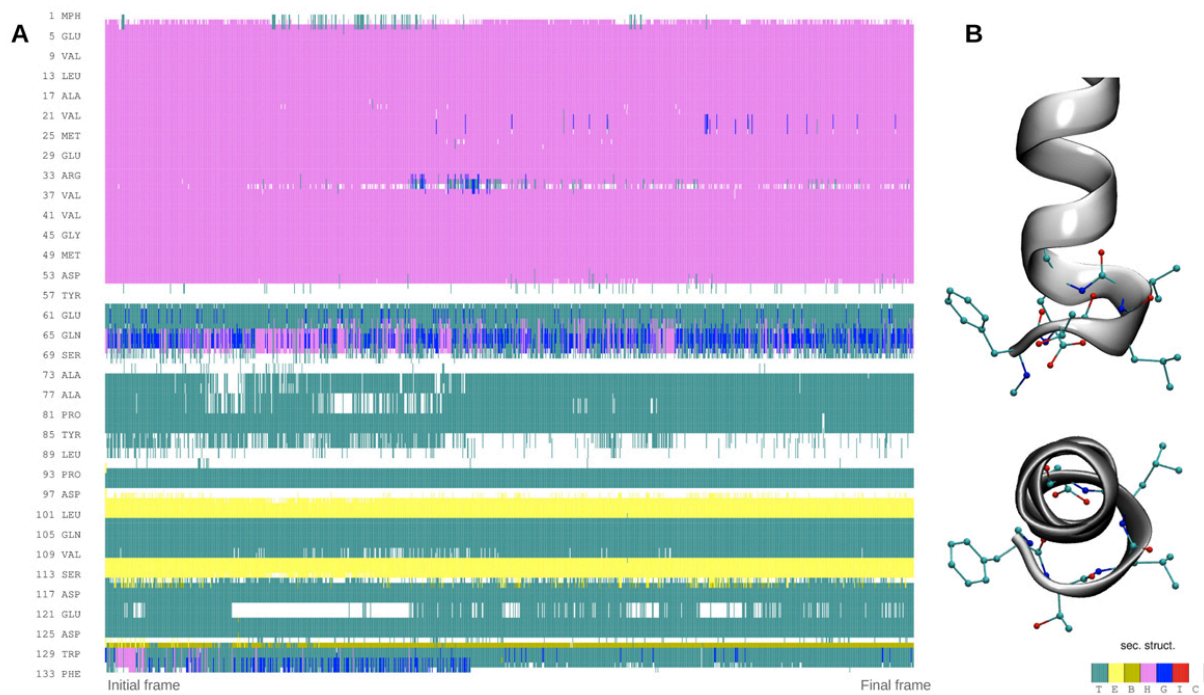
1028 indicated. For the  $\alpha$ -PulG blot only the band corresponding to PulG monomer is shown. Mw  
1029 markers and lane numbers are indicated. Uncropped images of  $\alpha$ -PulM and  $\alpha$ -PulG Western  
1030 blots of untreated and FA-treated samples are shown in Fig. S2.



1031

1032 **Fig. 7. Visualisation of MD simulations of PulG variants.**

1033 Snapshots of initial and representative final conformations of methylated MePulG<sup>WT</sup>,  
1034 methylated MePulG<sup>E5A</sup>, and non-methylated PulG<sup>E5A</sup> variants embedded in a POPE bilayer.  
1035 In all the simulations, the protein became more deeply buried, and in all (except PulG<sup>E5A</sup>  
1036 replica II) the protein bent towards the membrane surface, allowing the globular domain to  
1037 interact with the phospholipids. Changes in F1 and E5 did not cause severe protein  
1038 destabilization or membrane perturbation.



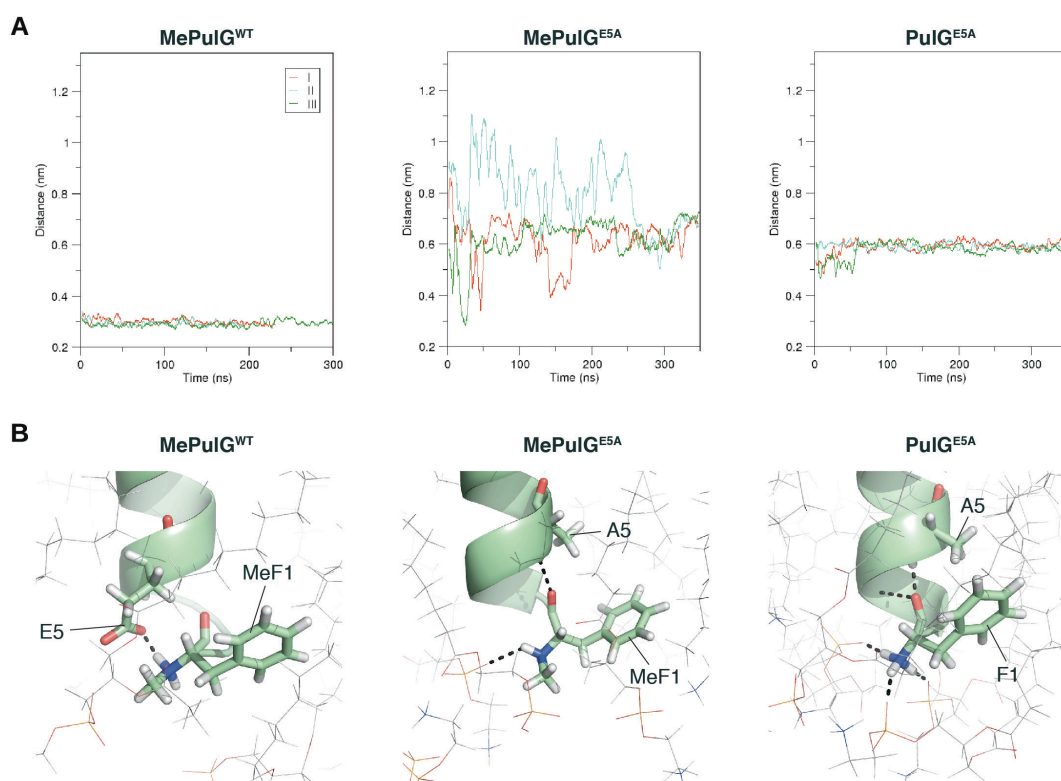
1039

1040 **Fig. 8. Secondary structure of PulG during MD simulations.**

1041 A. A representative data set showing the maintenance of protein secondary structure during a  
 1042 MePulG<sup>WT</sup> simulation. The graph shows the evolution of the secondary structure of each  
 1043 residue (y-axis) over time (x-axis), coloured as follows:  $\alpha$ -helix – purple; 3-10 helix – blue;  $\beta$ -  
 1044 sheet – yellow; turn – cyan; coil – white. In MePulG<sup>WT</sup> simulations the first several residues  
 1045 demonstrated a relaxation of helical structure into either turn or coil structures, suggesting that  
 1046 MeF1 and E5 destabilized the terminus as they deformed to interact with each other.

1047 B. The MeF1-E5 interaction caused the N-terminus to deform and created a loop, shown here  
 1048 with the protein backbone in grey and residues 1-5 shown as ball-and-stick structures,  
 1049 coloured by atom (carbon – cyan, oxygen – red, nitrogen – blue).





1050

1051 **Figure 9. N-terminal intra-molecular and protein-lipid interactions.**

1052 A. Graphs showing how the minimum distances between the residue 1 methyl group or amide  
 1053 terminus with any atom of the residue 5 side-chain varied during the simulations. In the  
 1054 MePulG<sup>WT</sup> system the methyl group of MeF1 and the E5 side-chain remained consistently  
 1055 within 3Å of each other, whereas in the mutant systems the distances were much larger. This  
 1056 suggested that MePulG<sup>WT</sup> E5 promotes intra-molecular interactions with the N-terminal  
 1057 positive charge of MeF1, anchoring PulG less firmly in the bilayer and priming the protein for  
 1058 extraction during pseudopilus assembly. In contrast, the N-terminus of the MePulG<sup>E5A</sup> and  
 1059 PulG<sup>E5A</sup> mutant variants interact with membrane lipids, anchoring the protein.

1060 B. Representative visualisations of hydrogen bonding of PulG N-terminus to POPE and  
 1061 solvent; the protein is shown as a green ribbon, with the labelled residues in stick format and  
 1062 coloured by atom (C – green, O – red, N – blue, H – white). Hydrogen bonds are shown as  
 1063 black dotted lines. POPE is depicted in stick format and coloured by atom (C – grey, O – red,  
 1064 N – blue, H – white, P – orange).

1065

1066

1067