

1 Pseudopilin residue E5 is essential for recruitment by the type 2 secretion system assembly  
2 platform

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23 Running title: Pseudopilin interactions with assembly factors

24 Key words: type 2 secretion system, type 4 pili, pilus assembly, bacterial two-hybrid,  
25 assembly platform, protein interactions

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27 **Summary**

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30 Type II secretion systems (T2SSs) promote secretion of folded proteins playing important  
31 roles in nutrient acquisition, adaptation and virulence of Gram-negative bacteria. Protein  
32 secretion is associated with the assembly of type 4 pilus (T4P)-like fibres called pseudopili.  
33 Initially membrane embedded, pseudopilin and T4 pilin subunits share conserved  
34 transmembrane segments containing an invariant Glu residue at the 5th position, E5.  
35 Mutations of E5 in major T4 pilins and in PulG, the major pseudopilin of the *Klebsiella* T2SS  
36 abolish fibre assembly and function. Among the four minor pseudopilins, only PulH required  
37 E5 for secretion of pullulanase, the substrate of the Pul T2SS. Mass-spectrometry analysis of  
38 pili resulting from the co-assembly of PulG<sup>E5A</sup> variant and PulG<sup>WT</sup> ruled out an E5 role in pilin  
39 processing and N-methylation. A bacterial two-hybrid analysis revealed interactions of the  
40 full-length pseudopilins PulG and PulH with the PulJ-PulI-PulK priming complex and with the  
41 assembly factors PulM and PulF. Remarkably, PulG<sup>E5A</sup> and PulH<sup>E5A</sup> variants were defective  
42 in interaction with PulM, but not with PulF and co-purification experiments confirmed the E5-  
43 dependent interaction between native PulM and PulG. These results reveal the role of E5 in  
44 a recruitment step critical for assembly of the functional T2SS, likely relevant to T4P  
45 assembly systems.

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## 49 **Introduction**

50

51 Bacterial interactions with their environment involve the transport of proteins and other  
52 macromolecules across their cell envelope. In Gram-negative bacteria, several sophisticated  
53 molecular machines facilitate protein secretion across two bilayer membranes (Costa *et al.*,  
54 2015). Among these, the type II secretion system (T2SS) promotes specific transport of  
55 folded proteins across the outer membrane once they have reached the periplasmic  
56 compartment through the Sec or the Tat systems (Voulhoux *et al.*, 2001; Korotkov *et al.*,  
57 2012; Nivaskumar and Francetic, 2014). T2SSs are structurally and functionally similar to  
58 type IV pilus (T4P) assembly systems (Berry and Pelicic, 2014). Unlike type IV pili, which  
59 extend beyond the bacterial surface to promote attachment and motility, the T2SS  
60 pseudopilus fibres are thought to be restricted to the periplasm. However, plate-cultured  
61 bacteria that overexpress T2SS-encoding genes present extended pseudopili (hereafter  
62 referred to as T2SS pili) on the cell surface, a feature that has facilitated their biochemical  
63 and structural characterisation (Sauvonnet *et al.*, 2000; Durand *et al.*, 2003; Kohler *et al.*,  
64 2004). T2SS pili are helical homo-polymers composed of one major pseudopilin subunit  
65 called PulG *in K. oxytoca* (Sauvonnet *et al.*, 2000; Kohler *et al.*, 2004). Mechanistic insights  
66 gained from their structure function analysis have revealed that interactions between  
67 neighbouring pilus subunits P and P<sup>+1</sup> tightly correlate with fibre assembly and function in  
68 protein secretion (Campos *et al.*, 2010). The fact that these essential contacts take place in  
69 the membrane implies a one-start helix rotational assembly mode for these and other type 4  
70 pilus-like fibres (Nivaskumar *et al.*, 2014).

71 Pseudopilus biogenesis is a multistep process that begins with co-translational  
72 insertion of pseudopilins in the inner membrane (IM) *via* the SRP and the Sec machinery  
73 (Arts *et al.*, 2007; Francetic *et al.*, 2007). Pseudopilins are made as precursors anchored in  
74 the IM through a positively charged N-terminal peptide that is removed prior to assembly by  
75 an inner membrane protease called the prepilin peptidase (Nunn and Lory, 1991). Following

76 cleavage of this peptide after a conserved Gly residue at the base of the transmembrane  
77 segment, the N-terminal domain of the prepilin peptidase transfers a methyl group to the new  
78 N-terminal residue of the mature pilin (Strom *et al.*, 1993; Aly *et al.*, 2013).

79 In addition to the highly abundant major pseudopilins that build the helical homo-  
80 polymeric fibre, T2SSs contain four minor pseudopilins, essential for function. Purified  
81 globular domains of three of these minor subunits, GspI, GspJ and GspK from  
82 enterotoxigenic *Escherichia coli* form a quasi-helical complex, which is predicted to cap the  
83 pseudopilus tip (Korotkov and Hol, 2008). Functional, biochemical and molecular dynamics  
84 analysis of their full-length equivalents in the Pul T2SS showed that Pull, PulJ and PulK self-  
85 assemble in the IM in the absence of other T2SS factors (Cisneros *et al.*, 2012a). The PulJ-  
86 Pull-PulK complex promotes initiation of pseudopilus assembly and presumably provides a  
87 template for subsequent incorporation of PulH and PulG subunits. Mutants lacking Pull, PulJ  
88 or PulK, but not PulH, assemble fewer PulG pili and are completely defective for secretion of  
89 the specific Pul T2SS substrate, pullulanase (PulA) (Cisneros *et al.*, 2012a). Biochemical  
90 studies of the *Pseudomonas aeruginosa* Xcp T2SS show that the periplasmic domain of the  
91 PulH homologue XcpU forms a complex with other minor pseudopilins *in vitro* by binding to  
92 the PulJ homologue XcpW (Yanez *et al.*, 2008; Korotkov and Hol, 2008; Douzi *et al.*, 2009).  
93 In addition, the major pseudopilin XpsG of *Xanthomonas campestris* T2SS and the minor  
94 subunit XpsH, a PulH homologue, interact directly *in vivo* (Hu *et al.*, 2002). Based on these  
95 studies, PulH homologues are thought to provide a link between the priming complex and the  
96 major pseudopilins (Yanez *et al.*, 2008; Korotkov and Hol, 2008; Douzi *et al.*, 2009; Cisneros  
97 *et al.*, 2012a).

98 Assembly of PulG fibres requires a functional complex called the assembly platform  
99 (AP) composed of the IM proteins PulC, PulF, PulL and PulM (Py *et al.*, 2001; Possot *et al.*,  
100 2000). The cytoplasmic domain of PulL binds the hexameric ATPase PulE that provides  
101 energy for pseudopilus assembly (Abendroth *et al.*, 2005; Camberg *et al.*, 2007).  
102 Biochemical and structural analyses of AP components in T2SSs have provided molecular  
103 insights into their soluble domains and hints about their interactions and organization

104 (Korotkov *et al.*, 2011; Abendroth *et al.*, 2004b; Abendroth *et al.*, 2004a; Sandkvist *et al.*,  
105 1995; Johnson *et al.*, 2007; Abendroth *et al.*, 2005). This information has led to the current  
106 T2SS model, where the IM platform promotes pseudopilus assembly within a cage-like  
107 compartment delimited by the secretin channel formed by a GspD dodecamer in the outer  
108 membrane and the GspD-interacting partner GspC in the IM (Gsp being a generic term to  
109 designate T2SS components) (McLaughlin *et al.*, 2012).

110         The hydrophobic segments of major (pseudo)pilins in bacterial T4P and T2SSs are  
111 highly conserved and rich in branched chain amino acid residues. In addition to the  
112 conserved Gly residue preceding the prepilin peptidase cleavage site, mature subunits  
113 contain an invariant Glu residue at position 5 (E5), which is located near the cytoplasmic side  
114 of the transmembrane segment and is essential for function (Strom and Lory, 1991; Aas *et al.*,  
115 2007; Pugsley, 1993). In the T4P systems, residue E5 has been implicated in prepilin  
116 processing (Pasloske and Paranchych, 1988) or N-methylation (Strom and Lory, 1991; Aas  
117 *et al.*, 2007). Based on structural studies of T4P, it has also been proposed that E5 is  
118 involved in docking of pilins to the growing fibre during assembly, by forming electrostatic  
119 contacts with the N-terminal amine of the last pilin subunit incorporated in the pilus (Parge *et al.*,  
120 1995; Craig *et al.*, 2006).

121         In contrast, the E5V substitution in PulG from *K. oxytoca* T2SS did not affect  
122 processing and N-methylation (Pugsley, 1993). Analyses of the PulG pilus structure and  
123 assembly show that E5 is not implicated in docking, but participates in fibre stabilisation.  
124 More precisely, once the protomer P has been extracted from the membrane, its E5 residue  
125 interacts with two lysine residues (K28 and K35) of protomer P<sup>-3</sup> (Campos *et al.*, 2010;  
126 Nivaskumar *et al.*, 2014). However, the Ala substitutions of these Glu and Lys residues lead  
127 to strikingly different phenotypes: while *pulG*<sup>K28A/K35A</sup> mutants are fully functional in protein  
128 secretion, *pulG*<sup>E5A</sup> mutants are completely defective. This and other data suggest an  
129 additional role for E5 in a step preceding the docking step in the pilus assembly pathway  
130 (Nivaskumar *et al.*, 2014). With the exception of the interactions between EpsG and the AP

131 component EpsL in *Vibrio cholerae* T2SS that have been revealed by chemical cross-linking  
132 (Gray *et al.*, 2011), little is known about the early events of the assembly process. To shed  
133 some light on these steps, we sought to characterise interactions between minor and major  
134 pseudopilins and to identify AP components that directly interact with pseudopilins using the  
135 *K. oxytoca* T2SS as a model system. By combining bacterial two-hybrid and co-purification  
136 approaches with mass spectrometry and functional analyses, we identified several  
137 interacting partners of the *K. oxytoca* major pseudopilin PulG and revealed a novel role of the  
138 conserved pseudopilin residue E5, which holds important clues to the assembly mechanism  
139 of the T4P fibre family.

140

## 141 **Results**

142

### 143 *Mass spectrometry analysis of PulG and PulG<sup>E5A</sup> co-assembled into mixed pili*

144

145 As mentioned above, earlier studies showed that residue E5 is not required for PulG  
146 processing or N-methylation (Pugsley, 1993), while studies of T4P suggest the opposite  
147 (Strom and Lory, 1991; Aas *et al.*, 2007). The assembly-defective pilin variants with E5  
148 residue substitutions have been shown to co-assemble into pili in the presence of wild type  
149 pilins (Pasloske, *et al.*, 1989; Aas *et al.*, 2007). However, while N-terminal sequencing of  
150 *Neisseria gonorrhoeae* T4P made of PilE wild type and E5L variants revealed under-  
151 methylation of the pilins, it was not clear whether this under-methylation was specific to  
152 PilE<sup>E5L</sup> (Aas *et al.*, 2007). To clarify the role of E5 in PulG, we used a similar approach and  
153 tested whether the assembly-defective PulG<sup>E5A</sup> variant can be co-assembled into pili with  
154 PulG<sup>WT</sup>. Therefore, we co-expressed the full set of genes encoding the Pul T2SS (on plasmid  
155 pCHAP8185, which includes PulG<sup>WT</sup>) with the gene encoding assembly-defective PulG<sup>E5A</sup>  
156 variant (on plasmid pCHAP7790, Table 1). The surface proteins were isolated from these  
157 bacteria and analysed by SDS-PAGE and Coomassie Blue staining (Experimental  
158 Procedures and Figure 1A). A prominent band of approximately 14.5 kDa, the molecular

159 mass of PulG, was present in the sheared fraction (Figure 1A, lane 1). This band was not  
160 present in samples from the negative control strain producing PulG<sup>E5A</sup> and the Pul T2SS  
161 lacking PulG ( $\Delta$ G, Figure 1A, lane 2). The intensity of this band was higher in the strain  
162 where both plasmids carried the wild type copy of the *pulG* gene (Figure 1A, lane 3),  
163 compared to the strain carrying a  $\Delta$ *pulG* version of T2SS complemented with wild type *pulG*  
164 (Figure 1A, lane 4). To test whether PulG<sup>E5A</sup> was co-assembled with PulG<sup>WT</sup>, the gel slices  
165 corresponding to the 14.5-kDa band were excised from lanes 1 (strain producing PulG<sup>WT</sup> and  
166 PulG<sup>E5A</sup>) and 4 (producing PulG<sup>WT</sup>) and analysed for protein content using top-down mass  
167 spectrometry (analysis of intact proteins without any digestion) (Gault *et al.*, 2014; Gault *et*  
168 *al.*, 2015). For PulG<sup>WT</sup> sample (Figure 1B), two major peaks were found at 14607.56 and  
169 14621.54 Da, corresponding to the molecular mass of PulG (theoretical Mr: 14607.51 Da)  
170 and methylated PulG (theoretical Mr: 14621.53 Da). For the PulG<sup>WT</sup> + PulG<sup>E5A</sup> sample  
171 (Figure 1C), two supplementary peaks corresponding to PulG<sup>E5A</sup> (experimental: 14549.49  
172 Da; theoretical: 14549.50 Da) and methylated PulG<sup>E5A</sup> (experimental: 14563.53 Da;  
173 theoretical: 14563.52 Da) were observed.

174 To confirm the N-terminal methylation and presence of an alanine residue (A) instead  
175 of a Glu (E) at the 5<sup>th</sup> position in the sequence, we performed High Energy Collision  
176 Dissociation (HCD) fragmentation of the (14<sup>+</sup>) ions of both methylated PulG and methylated  
177 PulG<sup>E5A</sup>. The MS/MS spectra are shown in Figure 2. The presence of series of b-type ions in  
178 both cases confirms without any ambiguity both the methylation of the N-terminus and the  
179 identity of the 5th amino acid.

180 These results show that PulG<sup>E5A</sup> can assemble into pili, provided it is co-produced  
181 with PulG<sup>WT</sup>. This suggests that PulG<sup>E5A</sup> likely co-assembles with native PulG into mixed pili.  
182 The presence of methylated and non-methylated forms of both PulG variants in the pili  
183 shows further that methylation of pseudopilins is not required for assembly.

184

185 *Differential requirements of residue E5 for the major and minor pseudopilin function*

186

187 With the exception of the PulK subunit, which caps the priming complex, all minor  
188 pseudopilins in T2SS have the conserved E5 residue. Given its strong conservation among  
189 (pseudo)pilins, we wondered whether E5 was also essential for minor pseudopilin function.  
190 To address this question, we introduced E5A substitutions in minor subunits PulH, Pull and  
191 PulJ and assessed their ability to support PulA secretion in conditions of low, chromosome-  
192 like levels of *pul* gene expression. The results of three independent secretion assays are  
193 shown in Figures 3A and supplementary Figure S1, and their quantification is shown in  
194 Figure 3B. Under these conditions, the PulG<sup>E5A</sup> variant was as defective for PulA secretion as  
195 the negative control strain lacking PulG (Figure 3A, lanes 1-6), as shown previously  
196 (Nivaskumar *et al.*, 2014). The *pulH* deletion mutant supported a low level of PulA secretion  
197 (Figure 3A, lanes 11,12) confirming that PulH is required for efficient secretion in  
198 physiological expression conditions (Cisneros *et al.*, 2012a). Surprisingly, PulA secretion in  
199 the presence of the PulH<sup>E5A</sup> variant was nearly abolished, suggesting a dominant negative  
200 effect of the *pulH*<sup>E5A</sup> allele (Figure 3A, lanes 7-12). In contrast, E5A substitutions in minor  
201 pseudopilins Pull and PulJ did not affect their function (Figure 3A, lanes 13-18 and 19-24).  
202 Replacing the Met-5 with Glu in variant PulK<sup>M5E</sup> had no effect on PulA secretion (Figure 3A,  
203 lanes 25-30), confirming previous results obtained in conditions of *pul* gene overexpression  
204 (Vignon *et al.*, 2003). Thus, the requirement for E5 appears to be specific to PulG and PulH  
205 pseudopilins, which are not part of the priming tip complex and have been implicated in the  
206 downstream steps of pseudopilus assembly (Cisneros *et al.*, 2012a).

207

### 208 *Pseudopilin interaction network*

209

210 The PulJ-Pull-PulK complex presumably provides a template for PulH incorporation  
211 (Cisneros *et al.*, 2012a), as suggested by the formation of the quaternary complex  
212 comprising all minor pseudopilin periplasmic domains in the *P. aeruginosa* Xcp T2SS (Yanez  
213 *et al.*, 2008; Korotkov and Hol, 2008; Douzi *et al.*, 2009). However, this complex did not  
214 interact with the globular domain of the major pseudopilin XcpT *in vitro* (Douzi *et al.*, 2009),



215 leaving the question of the mechanism that couples the initiation and elongation stages of  
216 assembly open. Based on the findings of Hu and collaborators (Hu *et al.*, 2002), it has been  
217 proposed that the minor pseudopilin quaternary complex and the major pseudopilin interact  
218 instead through their hydrophobic segments (Douzi *et al.*, 2009). To test this possibility and to  
219 characterise the pseudopilin interaction network, we employed the bacterial two-hybrid  
220 (BAC2H) approach that allows one to study interactions between full-length, membrane-  
221 embedded proteins (Karimova *et al.*, 1998). Using this method we had previously  
222 demonstrated interactions of Pull with PulJ and PulK (Cisneros *et al.*, 2012a). In the present  
223 study, we fused the T18 and T25 fragments of adenylyl cyclase CyaA from *Bordetella*  
224 *pertussis* to the N-terminus of all five full-length mature pseudopilins and performed BAC2H  
225 assays (Experimental Procedures). Using T18-PulG as bait, we identified strong interactions  
226 with two minor pseudopilins, PulH and PulJ (Figure 4). When T18-PulH was used as bait, a  
227 specific interaction was observed only with T25-PulG. Although both T18-PulH and T25-PulH  
228 chimera consistently showed a tendency to interact with PulJ, these interactions were not  
229 statistically significant despite low P values (0.106 and 0.201, respectively) (Figure 4A and  
230 Supplementary Dataset 1). The T18-Pull chimera showed specific interactions with PulJ and  
231 PulK (Figure 4), in agreement with previous studies in several T2SSs (Cisneros *et al.*, 2012a;  
232 Korotkov and Hol, 2008; Douzi *et al.*, 2009). Conversely, T18-PulJ interacted with Pull  
233 (Figure 4A). In addition, T18-Pull, T18-PulJ and T18-PulK showed a weak interaction with  
234 PulG. Of note, only PulG formed homo-dimers in the BAC2H assay, consistent with the  
235 unique ability of major pseudopilins to form long homo-polymers (Durand *et al.*, 2005). The  
236 results of these analyses, summarised schematically in Figure 4B, reveal multiple novel  
237 contacts between PulG and minor pseudopilins. Overall, the data are compatible with the  
238 formation of complexes containing all five pseudopilin species.

239

240 *Pseudopilin interactions with IM assembly platform components*

241

242 Previous functional studies suggest that the minor pseudopilin complex primes fibre  
243 assembly and that it might activate the ATPase PulE that is associated with the assembly  
244 platform (AP) complex (Cisneros *et al.*, 2012a). The AP is essential for T2SS function and  
245 contains multiple copies of IM proteins PulC, PulF, PulL and PulM (Py *et al.*, 2001; Possot *et*  
246 *al.*, 2000). To understand the physical and functional link between AP and the pseudopilin  
247 complex and to elucidate molecular details of the initiation and elongation steps of assembly,  
248 we assessed interactions between the pseudopilins and membrane-embedded AP  
249 components by using the BAC2H system. Therefore, we fused the T18- and T25- CyaA  
250 fragments to the N-terminal ends of full-length AP components PulC, PulF, PulL and PulM  
251 (Experimental Procedures). In the BAC2H assays, both T18-PulG and T25-PulG interacted  
252 specifically with the corresponding PulM- and PulF-CyaA chimera, but not with PulC-  
253 chimera. T18-PulG interacted very weakly with the T25-PulL, but the reciprocal pair did not  
254 interact (Figure 5A), which was surprising, given that their homologues in *V. cholerae*, EpsL  
255 and EpsG, interact in a cross-linking study (Gray *et al.*, 2011). We confirmed that this was  
256 not due to non-functional PulL chimera, since the latter did interact with corresponding PulM  
257 hybrids (Figure 5A, black bars on the right), as expected based on previous studies (Possot  
258 *et al.*, 2000; Buddelmeijer *et al.*, 2006). The results, summarized in Figure 5B, revealed  
259 strong and specific interactions of PulG with the PulM and PulF components of the assembly  
260 platform, suggesting their direct role in pseudopilus elongation.

261         Among the minor pseudopilins, PulH also interacted with PulF and PulM, although  
262 these interactions seem weaker than those observed between PulG and PulF or PulM,  
263 based on beta-galactosidase activity levels (Figure 5A). A weak "one-way" interaction was  
264 also detected between T18-PulK and T25-PulM chimera. In addition, PulF interacted weakly  
265 but significantly with Pull, PulJ and PulK in one out of two protein hybrid pairs. The high beta-  
266 galactosidase activity of strains producing T18-PulF and T25-PulF chimera in the BAC2H  
267 system (Figure 5A) shows a strong propensity of PulF for dimer formation.

268

269 *The PulG<sup>E5A</sup> and PulH<sup>E5A</sup> variants are specifically defective in the interaction with PulM*

270

271           Given the suggested role of E5A in an early step of the assembly process (Campos *et*  
272 *al.*, 2010; Nivaskumar *et al.*, 2014), we asked whether the E5A mutation affects interactions  
273 of PulG with one of the assembly factors. In BAC2H assay the PulG<sup>E5A</sup> variant showed equal  
274 or stronger interaction signal with PulF, compared to native PulG, as indicated by the high  
275 beta-galactosidase activities of T18-PulG<sup>E5A</sup> or T25-PulG<sup>E5A</sup> hybrids (Figure 6A). In contrast,  
276 the same PulG<sup>E5A</sup> hybrids showed a significantly decreased interaction with PulM (Figure  
277 6A). These results suggest that mutation E5A specifically alters the interface between PulG  
278 and PulM. Likewise, while the E5A substitution in PulH did not affect the PulH-PulF  
279 interaction, it reduced the interaction with PulM, supporting a role of E5 in PulM binding  
280 (Figure 6B).

281           The PulG-PulM interaction data were further validated with native, full-length proteins.  
282 We analysed PulG interactions with PulM and the effect of the PulG E5A substitution using a  
283 co-purification approach. PulG-His<sub>6</sub>, PulG<sup>E5A</sup>-His<sub>6</sub> or PulG<sup>WT</sup> were co-produced with PulM in  
284 the absence of other T2SS components (Experimental Procedures). Membrane proteins  
285 were extracted from these strains with Triton-X-100 and separated on the Ni-NTA columns.  
286 PulM was co-eluted with PulG-His<sub>6</sub> (Figure 7A, lanes 6-11). Despite the higher levels of the  
287 PulG<sup>E5A</sup>-His<sub>6</sub> variant produced and retained on the column, the quantity of co-purified PulM  
288 was significantly reduced (Figure 7A, lanes 17-22). Quantification of the relative amounts of  
289 PulG and PulM shows that the amount of co-eluted PulM was reduced nearly ten-fold,  
290 relative to PulG-His<sub>6</sub> (Figure 7B). As shown in Figure 7A, PulG and PulM were present in  
291 very low amounts in the elution fractions when membrane proteins were extracted from the  
292 control strain producing untagged PulG (lanes 28-33), indicating very low nonspecific binding  
293 to the Ni-NTA resin.

294           The results of these affinity co-purification experiments were in good agreement with  
295 those of the BAC2H analysis, revealing specific binding of PulM to PulG *in vivo*. They confirm  
296 that the E5A substitution in PulG, which abolishes pseudopilus assembly and protein  
297 secretion, specifically affected the interaction between PulG and PulM.

298

299 **Discussion**

300

301 Archaeal pili and flagella, as well as bacterial T2SS, T4P and competence systems use  
302 similar machineries to assemble helical fibres from membrane-anchored (pseudo)pilin  
303 subunits, suggesting a common mechanism. The globular (pseudo)pilin domains, exposed  
304 on the fibre surface, show variability in size and sequence, presumably optimized to promote  
305 specific functions (Berry and Pelicic, 2014; Nivaskumar and Francetic, 2014; Shahapure *et*  
306 *al.*, 2014). In contrast, their hydrophobic segments are highly conserved, which might reflect  
307 constraints imposed by their biogenesis and assembly pathway (Campos *et al.*, 2013). For  
308 example, high hydrophobicity is key for efficient targeting to the SRP and Sec pathways  
309 during integral membrane protein biogenesis (Francetic *et al.*, 2007; Arts *et al.*, 2007) and  
310 additional constraints might be linked to specific interactions with the prepilin peptidase or  
311 with fibre assembly factors. Here we used the *Klebsiella* T2SS as a model to study  
312 pseudopilus assembly, focusing on the most conserved (pseudo)pilin residue E5 playing an  
313 essential role in this process.

314         Although the E5 residue is involved in pseudopilus stabilisation via long-range  
315 interactions, genetic data suggest it plays a critical role at a step preceding pseudopilin  
316 docking. Using two complementary mass spectrometry methods we demonstrated  
317 unambiguously that PulG<sup>E5A</sup> co-assembles with PulG<sup>WT</sup> into mixed PulG pili containing the  
318 methylated and non-methylated forms of PulG and PulG<sup>E5A</sup>. This confirms previous findings  
319 that E5A substitution does not prevent N-methylation of PulG (Pugsley, 1993) and shows  
320 that methylation is not essential for pseudopilus assembly. This is in agreement with  
321 assembly of T4P in the methylase-deficient *pilD* mutants of *P. aeruginosa* (Pepe and Lory,  
322 1998) and with the small amount of non-methylated PulG detected in T2SS pilus  
323 preparations (Köhler *et al.*, 2004). Compared to PulG<sup>WT</sup>, the degree of N-methylated PulG<sup>E5A</sup>  
324 was lower, which might be a consequence of *pulG* overexpression increasing the load on the  
325 methyl-transferase function of the prepilin peptidase PulO. Of note, the proportion of PulG<sup>E5A</sup>

326 in the mixed pili was substantially lower than 50%, which could be explained by the  
327 dependence on PulG<sup>WT</sup> for assembly, as well as by the predicted impact of the E5A  
328 substitution on fibre stability, due to the loss of long-range interactions with K28<sup>P-3</sup> and K35<sup>P-3</sup>  
329 (Nivaskumar *et al.*, 2014). We propose that co-assembly of PulG<sup>E5A</sup> relies on its ability to  
330 form heterodimers with PulG<sup>WT</sup> *via* the previously characterised electrostatic contacts of  
331 conserved residues E44 and D48 of the incoming subunit (P<sup>-1</sup>) with R87 and R88 of the P  
332 subunit incorporated in the PulG pilus (Campos *et al.*, 2010; Nivaskumar *et al.*, 2014). This  
333 association with PulG<sup>WT</sup> in the membrane would bypass the defect of PulG<sup>E5A</sup> possibly  
334 related to its targeting to the assembly site, as discussed below.

335 Pseudopilus assembly requires the formation of the PulJ-Pull-PulK priming complex,  
336 essential for protein secretion by the T2SS (Cisneros *et al.*, 2012a). While our previous  
337 studies demonstrated the correlation between the formation of this complex and pseudopilus  
338 assembly activation (Cisneros *et al.*, 2012a; Cisneros *et al.*, 2012b), the molecular events  
339 linking the two processes have not been identified. To understand this link, we employed the  
340 BAC2H approach and characterised the network of interactions between full-length  
341 membrane-embedded pseudopilins and between individual pseudopilins and the AP  
342 components. The results confirmed the Pull-PulJ and Pull-PulK interactions observed *in vitro*  
343 in T2SSs from *Vibrio* (Yanez *et al.*, 2008), *E. coli* (Korotkov and Hol, 2008) and *P.*  
344 *aeruginosa* (Douzi *et al.*, 2009). In the BAC2H assay, the full-length PulH showed only a  
345 weak tendency to interact with PulJ, although the periplasmic domains of their *Pseudomonas*  
346 homologues interacted *in vitro* (Douzi *et al.*, 2009). In addition, contrary to the latter study, we  
347 identified direct contacts of PulG with PulH, which are in agreement with the results obtained  
348 in *Xanthomonas* T2SS studies (Hu *et al.*, 2002). In the *P. aeruginosa* T4P, the BAC2H  
349 analysis revealed very similar interactions between FimU, the PulH structural homologue,  
350 and the major pilin PilA, showing that these contacts are conserved in both systems (Nguyen  
351 *et al.*, 2015). Moreover, in the same study, FimU also failed to interact with FimW, the PulJ  
352 equivalent in this T4P system. Finally, we also identified novel direct contacts of PulG with  
353 PulJ that are in good agreement with the proposed structural models wherein GspH, but also

354 GspG, could be docked directly to GspJ at the base of the tip complex (Korotkov and Hol,  
355 2008). An interaction between PulG and PulJ might bypass the requirement for PulH,  
356 explaining the minor effect of *pulH* deletion on PulG pilus assembly (Cisneros *et al.*, 2012a)  
357 and on PulA secretion (Sauvonnet *et al.*, 2000) in conditions of *pul* gene overexpression.  
358 However, when the T2SS components are not overproduced, this requirement becomes  
359 more critical, consistent with the strongly defective PulA secretion in the absence of PulH  
360 (Figure 3). In addition, all minor pseudopilin subunits showed a weak tendency to interact  
361 with PulG in the BAC2H assay, which might explain the capacity of Pull-PulJ and Pull-PulK  
362 minor pseudopilin pairs to partially restore initiation of PulG assembly in  $\Delta pulHIJK$  mutants  
363 (Cisneros *et al.*, 2012a).

364         How do pseudopilins connect to the AP and with the associated ATPase, essential for  
365 fibre elongation? The results of our BAC2H analysis show that PulH and PulG, but not the  
366 other minor pseudopilins, interact specifically with two AP components, PulM and PulF,  
367 which might provide a basis for the recruitment of the initiation complex to the assembly site.  
368 We demonstrated that PulG and PulH interact with the conserved AP component PulF,  
369 presumably *via* TM segments of the two partners, given the absence of prominent  
370 periplasmic segments in PulF. In view of their high conservation, PulF and its equivalents in  
371 T4P assembly systems might be the main factors imposing restraints on the pilin primary  
372 sequence. The sequence conservation of pseudopilin TM segments is consistent with weak  
373 but significant binding of all minor pseudopilins to PulF in the BAC2H assay (Figure 5).  
374 Although we cannot exclude the possibility that these weak interactions anchor the PulJ-I-K  
375 priming complex to PulF, we favour the model wherein the quaternary minor pseudopilin  
376 complex binds to PulF via PulH. In addition, PulF showed a strong tendency to form dimers  
377 in this assay, consistent with the crystallography data (Abendroth *et al.*, 2009) and with the  
378 observed oligomerisation of its homologue PilC from *Thermus thermophilus* (Karuppiah *et*  
379 *al.*, 2010). In the recent study of the *Myxococcus xanthus* T4P architecture by cryo-electron  
380 tomography, the PulF homologue PilC also appears to form a dimer (Chang *et al.*, 2016).  
381 During fibre elongation, PulF dimers could act as membrane scaffolds or docking sites for the

382 minor pseudopilin complex (or the nascent pilus) on one hand, and the incoming PulG  
383 subunits on the other, to facilitate assembly. In the large-scale BAC2H interaction study of  
384 T4P from *Neisseria meningitidis*, the major pilin PilE interacted with the PulF homologue  
385 PilG, and both PilE and PilG formed homodimers, much like PulG and PulF (Georgiadou *et*  
386 *al.*, 2012). In the *P. aeruginosa* and *Myxococcus xanthus* T4P, the PulF homologue called  
387 PilC interacts with the ATPase PilB (Takhar *et al.*, 2013; Bischof *et al.*, 2016), consistent with  
388 its role in transduction of mechanical energy generated by the ATPase. In the *E. coli* bundle-  
389 forming pili (Bfp) the ATPase activity of BfpD is stimulated by specific regions of the PulF  
390 homologue BpfE (Crowther *et al.*, 2005).

391         Importantly, the BAC2H results revealed interactions of PulG and PulH with the PulM  
392 component of the AP. Moreover, the direct interaction of native PulG and PulM was  
393 confirmed by a complementary co-purification approach. So far, PulM has been implicated in  
394 stabilization of Pull within the AP complex (Possot *et al.*, 2000). While the results of the  
395 BAC2H analysis confirm PulM interaction with Pull, widely observed and well-studied in  
396 other T2SSs (Py *et al.*, 2001; Johnson *et al.*, 2007; Lallemand *et al.*, 2013), the strong and  
397 specific interactions with PulG and PulH reveal a novel role for PulM, possibly in pseudopilin  
398 recruitment to the assembly complex. Furthermore, we have shown that E5A substitutions in  
399 PulG and PulH interfere with their interactions with PulM in the absence of any other Pul  
400 factors, which implies that, in both cases, the E5 residue is a key feature of the PulG-PulM  
401 and PulH-PulM interfaces. These interaction defects caused by the E5A substitution likely  
402 account for the dramatic assembly defect of PulG<sup>E5A</sup> variant. The trans-dominant effect of the  
403 *pulH*<sup>E5A</sup> allele might be due to the reduced ability of PulH<sup>E5A</sup> to target the priming complex to  
404 PulM, as discussed below. In contrast, residue E5 is dispensable for function of Pull and PulJ  
405 subunits, although it might provide optimal packing with the N-termini of the distal  
406 neighbouring pseudopilins in the complex, as proposed previously (Craig *et al.*, 2003).

407         Our results show that PulG interacts strongly and specifically with PulM, but only very  
408 weakly with Pull, which apparently contradicts the studies in *Vibrio*, showing that EpsG could  
409 be cross-linked with EpsL (Gray *et al.*, 2011). This difference in interaction might reflect

410 differences between the *Vibrio* and *Klebsiella* T2SSs, or could be due to a difference in the  
411 techniques used to detect G-L interactions. The interaction of EpsG with EpsL was observed  
412 using chemical cross-linking, which might enhance and stabilize a weak or indirect  
413 association (Gray *et al.*, 2011). Here, we identified direct associations of PulG with PulM and  
414 PulF in the absence of other T2SS components using BAC2H, and also, in the case of PulG  
415 and PulM, by co-purification in the presence of nondenaturing detergents. Nevertheless, both  
416 studies support a similar model wherein interactions of major pseudopilin with the L-M  
417 complex result in pilin recruitment to the assembly site.

418 The architecture of the T2SS, proposed based on partial high- and low-resolution  
419 structural data of individual components (McLaughlin *et al.*, 2012) is very similar to that of the  
420 *Thermus thermophilus* or *Myxococcus xanthus* T4P determined by cryo-electron tomography  
421 (Gold *et al.*, 2015; Chang *et al.*, 2016). In all models, the secretin and the AP components  
422 homologous to PulC, PulL and PulM delimit a cage-like compartment that encloses PulF  
423 within and connects directly with the ATPase PulE at its base. While cryoEM tomography  
424 shows that the pilus occupies the interior of this compartment, PilO (a PulM homologue) and  
425 its interacting partner PilN (corresponding to the transmembrane and periplasmic domains of  
426 Pull) were placed in the lower periplasmic ring surrounding the cavity (Chang *et al.*, 2016).

427 Integrated with the previous data, the results of this study allow us to propose a  
428 schematic working model of pseudopilus assembly initiation and elongation (Figure 8). In this  
429 model, one of the initial steps, following pseudopilin maturation by the prepilin peptidase,  
430 would be the assembly of the priming complex PulJ-Pull-PulK and its binding to PulH and  
431 PulG (step 1). Consistent with the direct binding of PulH and PulG to PulF, and with PulF  
432 self-interaction, this complex would associate with a dimer of PulF (step 2). In step 3, the  
433 pseudopilins and PulF would bind to PulM, outside or within the pre-assembled complex that  
434 includes the secretin PulD channel in the OM bound to the IM components PulC and Pull.  
435 An independent assembly of this latter complex is consistent with the results of cellular  
436 localisation studies of T2SS components fused to fluorescent protein tags (Buddelmeijer *et al.*  
437 *et al.*, 2009; Lybarger *et al.*, 2009), as well as with the T4P cryo-electron tomography data



438 (Chang *et al.*, 2016). Importantly, association of PulM with PulF-pseudopilin complex  
439 requires residue E5 of PulG and PulH (Figure 8, step 3). Results of the T4P study by cryo-  
440 tomography suggest that the ATPase is recruited to the assembly site in the final step of the  
441 T4P complex biogenesis (Chang *et al.*, 2016). A similar order of assembly, in which the  
442 incorporation of the PulF-pseudopilin complex into the incomplete T2S machine would  
443 precede the recruitment of the ATPase PulE, is possible in T2SS (steps 4 and 5). However,  
444 there is an important difference between the two systems at the level of PulL, which  
445 corresponds to a fusion between PilM and PilN of T4P, leaving open a possibility that  
446 ATPase recruitment occurs independently of PulF.

447         During pseudopilus elongation, PulG, possibly in the form of dimers, would be  
448 recruited to the assembly site *via* interaction with PulM, through the critical residue E5 (step  
449 6). PulG would dock to the available PulF "acceptor" site and bind to the membrane-  
450 embedded PulG<sup>P+1</sup> subunit incorporated in the pseudopilus. ATP hydrolysis could drive  
451 rotation of the pilus *via* PulF to spool the protomer P into the growing fibre (step 7), following  
452 the one-start helix assembly model that we proposed and described earlier (Campos *et al.*,  
453 2010; Nivaskumar *et al.*, 2014). Fibre elongation would comprise cycles of targeting, docking  
454 and spooling events (steps 6 to 9).

455         Based on this model, we propose that co-assembly of PulG<sup>E5A</sup> relies on its ability to  
456 form heterodimers with PulG<sup>WT</sup>, which would promote targeting to the assembly site  
457 (Campos *et al.*, 2010; Nivaskumar *et al.*, 2014). The trans-dominant effect of PulH<sup>E5A</sup> variant  
458 would be due to its association to pseudopilin-PulF complex but not to PulM. While providing  
459 testable hypotheses, this model raises numerous questions, notably those related to the  
460 mechanism of exoprotein substrate recruitment and transport. Whether a recruitment step  
461 accompanies uptake of exoprotein substrate molecules, or whether there are mechanisms  
462 that control substrate entry to the preformed assembly site, are only some of the questions  
463 that need to be addressed by further investigation.

464         The interaction network of pseudopilin subunits and assembly platform proteins in the  
465 inner membrane described here will provide the basis for further biochemical and structure

466 function analysis of pseudopilus biogenesis and its mechanistic link with T2SS-mediated  
467 protein secretion.

468

## 469 **Experimental Procedures**

470

### 471 *Bacterial strains and plasmids*

472

473 The *Escherichia coli* strain DH5 $\alpha$  [*F'* *lacI*<sup>Q</sup>  $\Delta$ *lacZM15 pro+* *Tn10*)] was used for recombinant  
474 DNA experiments. Strains PAP7460 [ $\Delta$ (*lac-argF*)*U169 araD139 relA1 rpsL150  $\Delta$ malE444*  
475 *malG501*] and PAP5207 [ $\Delta$ (*lac-argF*)*U169 araD139 relA1 rpsL150 pcnB::Tn10 (F' lacI*<sup>Q</sup>  
476  *$\Delta$ lacZM15 pro+)*] were used for *pul* gene expression studies. The  $\Delta$ *cyo* strain DHT1 (Dautin  
477 *et al.*, 2000) was used for bacterial two-hybrid experiments. The bacteria were grown in LB  
478 medium supplemented with antibiotics, as required: chloramphenicol (Cm) (25  $\mu$ g ml<sup>-1</sup>),  
479 ampicillin (Ap) (100  $\mu$ g ml<sup>-1</sup>) or kanamycin (Km) (15 or 25  $\mu$ g ml<sup>-1</sup>). The expression of the *pul*  
480 genes was induced by addition of 0.4 % maltose. Isopropyl- $\beta$ -D-thiogalactoside was added to  
481 induce *lacZ* promoter-controlled gene expression in strain PAP5207.

482

### 483 *Plasmid construction*

484

485 Plasmids used in this study are listed in Table 1. Plasmid DNA purification, gel extraction and  
486 PCR product purification were performed using appropriate Qiagen kits. Restriction  
487 enzymes, DNA ligase and other molecular biology reagents were purchased from Fermentas  
488 or New England Biolabs. The high-fidelity Pwo polymerase (Roche) was used for PCR  
489 amplification and site directed mutagenesis using the modified Quick-change method. The  
490 list of oligonucleotides (synthesized by Sigma Genosys) used for cloning or site-directed  
491 mutagenesis is provided in Supplementary Table 1. To construct the BAC2H chimera, the *pul*  
492 genes were PCR-amplified using corresponding primers, treated with DpnI and cloned into

493 pKT25 and pUT18c vectors using *KpnI* and *EcoRI* enzymes. All plasmids were sequenced  
494 by GATC.

495

#### 496 *Bacterial two-hybrid and statistical analysis*

497

498 Competent cells of strain DHT1 were co-transformed with pUT18C and pKT25 derivatives  
499 and bacteria were grown for 48 h at 30°C on LB plates containing Ap and Km. Colonies were  
500 picked at random and inoculated into 5 ml cultures in LB containing Km and Ap, grown  
501 overnight and inoculated the next day into fresh medium containing 1 mM IPTG. Bacteria  
502 were cultured to mid-log phase and  $\beta$ -galactosidase activity was measured as described  
503 (Miller, 1972). At least 2 independent experiments were performed with 3 randomly picked  
504 transformants. Mean values were presented by bar graphs, and error bars indicate standard  
505 deviation. Microsoft Excel software was used for data processing and presentation. The  
506 statistical analysis was performed using the non-parametric Kruskal-Wallis test, followed by  
507 Dunn's post-test for multiple comparisons, using the Prism software.

508

#### 509 *Purification of PulG pili*

510

511 PAP7460 bacteria were transformed with pCHAP8185 plasmid containing all *pul* T2SS  
512 genes including *pulG<sup>WT</sup>*, or its derivative pCHAP8184 lacking *pulG*, and with pSU18 or its  
513 derivatives pCHAP1205 (*PulG<sup>WT</sup>*) or pCHAP7790 (*PulG<sup>E5A</sup>*) carrying different *pulG* alleles  
514 under control of the *lacZ* promoter. Bacteria were cultured on LB agar containing Ap, Cm and  
515 0.4 % maltose. After incubating for 48 h at 30°C, bacteria were harvested and resuspended  
516 in LB medium. Pili were sheared by vortex treatment and 20 passages through a 26-Gauge  
517 needle. Two consecutive centrifugation steps at 16 000 g for 20 min were used to separate  
518 bacteria and pili. The pilus fractions were further collected by ultracentrifugation in rotor Ti60  
519 at 150 000 g and pili were resuspended in 20 mM HEPES for further analysis by SDS-PAGE  
520 and mass spectrometry.

521

522 *Mass spectrometry*

523

524 All samples were desalted by C<sub>4</sub> ZipTip® (Millipore) and eluted directly into a 10 µL spray  
525 solution of methanol:water:formic acid (75:25:3). Between 2 and 6 µL were introduced into  
526 an Orbitrap Velos mass spectrometer, equipped with ETD module (Thermo Fisher Scientific,  
527 Bremen, Germany) using a TriVersa NanoMate® (Advion) in positive ion mode. The spray  
528 voltage was set to 1.2-1.6 kV and back-pressure to 0.3-0.4 psi. A full set of automated  
529 positive ion calibrations was performed immediately prior to mass measurement. The  
530 transfer capillary temperature was lowered to 100°C, sheath and auxiliary gasses switched  
531 off and source transfer parameters optimised using the auto tune feature. Helium was used  
532 as the collision gas in the linear ion trap. The FT automatic gain control was set at  $1 \times 10^6$  for  
533 MS experiments. Spectra were acquired in the FTMS in full profile mode with between 10  
534 microscans over several minutes, with averaging on and set to max, and a resolution of  
535 60,000 at 400 m/z. The final few spectra were then averaged using Qualbrowser in Thermo  
536 Xcalibur 2.1 and deconvoluted using Xtract to produce zero charge mass spectra.

537 For MS/MS experiments, the FT automatic gain control was set at  $2 \times 10^5$ . Ions corresponding  
538 to the isotopic distribution of a single charge state ( $14^+$ ) were selected with the largest  
539 possible window to avoid overlap with neighbouring species but minimize signal loss. HCD  
540 was performed at 27 eV and spectra were acquired in the FTMS in full profile mode at a  
541 resolution of 60,000 at 400 m/z, with 10 microscans and with averaging on and set to the  
542 maximum value. The final few spectra were then averaged using Qualbrowser in Thermo  
543 Xcalibur 2.1 and deconvoluted using Xtract to produce singly charged MS/MS spectra.  
544 MS/MS spectra were interpreted manually.

545

546 *SDS-PAGE and immuno-detection*

547

548 Proteins from bacterial extracts were separated by electrophoresis on 9 % or 10 %  
549 polyacrylamide gels and transferred onto nitrocellulose membranes (ECL, Amersham) using  
550 a semi-dry blotting apparatus. Membranes were blocked with 5% milk in TBST (10 mM Tris,  
551 150 mM NaCl, 0.05% Tween 20) and incubated with polyclonal antisera raised against  
552 purified PulA, PulG and MalE-PulM (diluted 1:1000 in TBST-5% milk), followed by four 10-  
553 min washes and incubation in horseradish peroxidase-coupled anti-rabbit antibody (1:40,000;  
554 Amersham). Membranes were developed by enhanced chemiluminescence ECL2 (Thermo)  
555 or Western Lightning Plus ECL (PerkinElmer) and recorded using the Typhoon phosphor-  
556 imager (GE) or LAS 4000 imager (Fujifilm). ImageJ software (Abramoff *et al.*, 2004) was  
557 used to quantify the density of bands.

558

#### 559 *PulA secretion assay*

560

561 PAP5207 bacteria were transformed with pCHAP8185 plasmid derivatives containing *pul*  
562 genes with corresponding pseudopilin gene knockouts, and a pSU18 empty vector or its  
563 derivatives containing the missing wild type or mutant pseudopilin gene (Table 1). Bacteria  
564 were grown in LB containing Ap and Cm in the presence of 0.4% maltose, 1/10 volume of M9  
565 salts and 1 mM IPTG to early stationary phase ( $OD_{600nm} > 2$ ). Cultures were normalized to  
566  $OD_{600nm} = 1$  and bacteria were pelleted by centrifugation for 10 min at 16000 g at 4°C.  
567 Bacteria were resuspended in SDS-sample buffer at final concentration of 1  $OD_{600nm}$  per ml.  
568 Supernatant fractions were centrifuged again for 10 min and mixed with equal volume of 2x  
569 SDS-sample buffer. Samples corresponding to 0.05  $OD_{600nm}$  from each fraction were  
570 separated by SDS-PAGE on 9% Tris-Tricine gels. Proteins were transferred to nitrocellulose  
571 membranes (ECL Amersham) by semi-dry electro-transfer. Polyclonal antisera were used for  
572 immuno-detection of PulA.

573

#### 574 *Co-purification of PulG-bound proteins*

575

576 Bacteria of strain PAP7460 producing PulG-His<sub>6</sub>, PulG<sup>E5A</sup>-His<sub>6</sub> or PulG<sup>WT</sup> variants encoded  
577 by pCHAP1362, pCHAP7785 and pCHAP8658, respectively and PulM (from plasmid  
578 pCHAP2393) were cultured at 30°C in LB medium containing Cm and Ap to OD<sub>600nm</sub> = 1.  
579 Bacteria were collected by centrifugation and broken by sonication in cold TBS (20 mM Tris-  
580 HCl, pH 7.4, 150 mM NaCl) with 100 µg.ml<sup>-1</sup> of lysozyme. The lysate was cleared by  
581 centrifugation to remove unbroken debris. Membranes were collected by ultracentrifugation  
582 at 186000 x g and resuspended in cold TBS, followed by solubilisation with 2% Triton X-100.  
583 Ultracentrifugation was repeated at 150000 g to remove non-solubilised membranes. Ni-NTA  
584 resin beads were washed with ~10 volumes of TBS and the solubilised membrane fraction  
585 was incubated with Ni-NTA resin at 4°C. The flow-through was collected, followed by seven  
586 washes with two column volumes of TBS supplemented with 20 mM imidazole. Proteins  
587 were eluted with TBS containing 300 mM imidazole.

588

## 589 **Acknowledgments**

590

591 We thank Evelyne Richet for insightful comments and for the critical reading of the  
592 manuscript, Jenny-Lee Thomassin for the help with statistical analysis, Alexandra East and  
593 Peter J. Bond for helpful suggestions. We are grateful to all members of the Laboratory of  
594 Macromolecular Systems and Signaling and of the Laboratory of Intercellular Communication  
595 and Microbial Infections for helpful discussions and friendly support. We thank Gouzel  
596 Karimova and Daniel Ladant for strains, plasmids and advice concerning the BAC2H  
597 analysis. This work was funded by the ANR FiberSpace grant N°ANR-14-CE09-0004. MN  
598 was a scholar of the Pasteur-Paris University (PPU) International PhD Program. JSM is  
599 funded by a fellowship from the Basque Government. JCR and CM acknowledge the DIM  
600 MALINF from the Ile de France Region for funding the LTQ Orbitrap Velos. The authors  
601 declare that they have no conflict of interest.

602

## 603 **References**

- 605 Aas, F. E., H. C. Winther-Larsen, M. Wolfgang, S. Frye, C. Løvold, N. Roos, *et al.*, (2007)  
 606 Substitutions in the N-terminal alpha helical spine of *Neisseria gonorrhoeae* pilin  
 607 affect Type IV pilus assembly, dynamics and associated functions. *Mol Microbiol.* **63**:  
 608 69-85.
- 609 Abendroth, J., M. Bagdasarian, M. Sandkvist and W. G. Hol, (2004a) The structure of the  
 610 cytoplasmic domain of EpsL, an inner membrane component of the type II secretion  
 611 system of *Vibrio cholerae*: an unusual member of the actin-like ATPase superfamily. *J*  
 612 *Mol Biol* **344**: 619-633.
- 613 Abendroth, J., D. Mitchell, K. Korotkov, T. Johnson, A. Kreger, M. Sankvist, *et al.*, (2009) The  
 614 three-dimensional structure of the cytoplasmic domains of EpsF from the type 2  
 615 secretion system of *Vibrio cholerae*. *J Struct Biol* **166**: 303-315.
- 616 Abendroth, J., P. Murphy, M. Sandkvist, M. Bagdasarian and W. G. Hol, (2005) The X-ray  
 617 structure of the type II secretion system complex formed by the N-terminal domain of  
 618 EpsE and the cytoplasmic domain of EpsL of *Vibrio cholerae*. *J Mol Biol* **348**: 845-  
 619 855.
- 620 Abendroth, J., A. E. Rice, K. McLuskey, M. Bagdasarian and W. G. Hol, (2004b) The crystal  
 621 structure of the periplasmic domain of the type II secretion system protein EpsM from  
 622 *Vibrio cholerae*: the simplest version of the ferredoxin fold. *J Mol Biol* **338**: 585-596.
- 623 Abramoff, M. D., P. J. Magelhaes and S. J. Ram, (2004) Image Processing with ImageJ.  
 624 *Biophotonics Internatl* **11**: 36-42.
- 625 Aly, K., E. Beebe, C. Chan, M. Goren, C. Sepúlveda, S. Makino, *et al.*, (2013) Cell-free  
 626 production of integral membrane aspartic acid proteases reveals zinc-dependent  
 627 methyltransferase activity of the *Pseudomonas aeruginosa* prepilin peptidase PilD.  
 628 *Microbiologyopen* **2**: 94-104.
- 629 Arts, J., R. van Boxtel, A. Filloux, J. Tommassen and M. Koster, (2007) Export of the  
 630 pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the  
 631 signal recognition particle-Sec pathway. *J Bacteriol* **189**: 2069-2076.
- 632 Bartolomé, B., Y. Jubete, E. Martinez and F. de la Cruz, (1991) Construction and properties  
 633 of a family of pACYC184-derived cloning vectors compatible with pBR322 and its  
 634 derivatives. *Gene* **102**: 75-78.
- 635 Berry, J.-L. and V. Pelicic, (2014) Exceptionally widespread filaments composed of type IV  
 636 pilins: the prokaryotic Swiss Army knives. *FEMS Microbiol Rev* **39**: 134-154.
- 637 Bischof, L. F., C. Friedrich, A. Harms, L. Søgaard-Andersen and C. van der Does, (2016)  
 638 The type IV pilus assembly ATPase PilB of *Myxococcus xanthus* interacts with the  
 639 inner membrane platform protein PilC and the nucleotide binding protein PilM. *J Biol*  
 640 *Chem* **291**: 6946-57.
- 641 Buddelmeijer, N., O. Francetic and A. Pugsley, (2006) Green fluorescent chimeras indicate  
 642 nonpolar localization of pullulanase secretion components PulL and PulM. *J Bacteriol.*  
 643 **188**: 2928-2935.
- 644 Buddelmeijer, N., M. Krehenbrink, F. Pecorari and Pugsley, A.P. (2009) Type II secretion  
 645 system secretin PulD localizes in clusters in the *Escherichia coli* outer membrane. *J.*  
 646 *Bacteriol.* **191**: 161-168.
- 647 Camberg, J. L., T. L. Johnson, M. Patrick, J. Abendroth, W. G. Hol and M. Sandkvist, (2007)  
 648 Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids.  
 649 *The EMBO journal* **26**: 19-27.
- 650 Campos, M., D. Cisneros, M. Nivaskumar and O. Francetic, (2013) The type II secretion  
 651 system - a dynamic fiber assembly nanomachine. *Res Microbiol* **164**: 545-555.
- 652 Campos, M., M. Nilges, D. A. Cisneros and O. Francetic, (2010) Detailed structural and  
 653 assembly model of the type II secretion pilus from sparse data. *Proc Natl Acad Sci U*  
 654 *S A.* **107**: 13081-13086.
- 655 Chang, Y.-W., Rettberg, L.A., Treuner-Lange, A., Iwasa, J., Sogaard-Andersen, L. and  
 656 Jensen, G. (2016) Architecture of the type IVa pilus machine. *Science* **351**: aad2001.

657 Cisneros, D. A., P. J. Bond, A. P. Pugsley, M. Campos and O. Francetic, (2012a) Minor  
658 pseudopilin self-assembly primes type II secretion pseudopilus elongation. *The*  
659 *EMBO journal* **31**: 1041-1053.

660 Cisneros, D.A., Péhaut-Arnaudet, G. and O. Francetic, (2012b). Heterologous assembly of  
661 type IV pili by a type II secretion system reveals the role of minor pilins in assembly  
662 initiation. *Mol. Microbiol.* **84**: 805-818.

663 Costa, T. R. D., C. Felisberto-Rodrigues, A. Meir, M. S. Prevost, A. Redzej, M. Trokter, *et al.*,  
664 (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic  
665 insights. *Nat Microbiol Rev* **13**: 343-359.

666 Craig, L., R. Taylor, M. Pique, B. Adair, A. Arvai, M. Singh, *et al.*, (2003) Type IV pilin  
667 structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated  
668 pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol Cell* **11**: 1139-1150.

669 Craig, L., N. Volkmann, A. S. Arvai, M. E. Pique, M. Yeager, E. H. Egelman, *et al.*, (2006)  
670 Type IV pilus structure by cryo-electron microscopy and crystallography: implications  
671 for pilus assembly and functions. *Mol Cell.* **23**: 651-662.

672 Crowther, L.J.A. Yamagata, L. Craig, J.A. Tainer and M.S. Donnenberg. (2005) The ATPase  
673 activity of BfpD is greatly enhanced by zinc and allosteric interactions with other Bfp  
674 proteins. *J. Biol. Chem.* **280**: 24839-24848.

675 Dautin, N., G. Karimova, A. Ullman and D. Ladant, (2000) Sensitive genetic screen for  
676 protease activity based on a cyclic AMP signaling cascade in *Escherichia coli*. *J*  
677 *Bacteriol* **182**: 7060-7066.

678 Douzi, B., E. Durand, C. Bernard, S. Alphonse, C. Cambillau, A. Filloux, *et al.*, (2009) The  
679 XcpV/Gspl pseudopilin has a central role in the assembly of a quaternary complex  
680 within the T2SS pseudopilus. *J Biol Chem* **284**: 34580-34589.

681 Durand, E., A. Bernadac, G. Ball, A. Lazdunski, J. Sturgis and A. Filloux, (2003) Type II  
682 protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar and  
683 adhesive structure. *J Bacteriol* **185**: 2759-2758.

684 Durand, E., G. Michel, R. Voulhoux, J. Kurner, A. Bernadac and A. Filloux, (2005) XcpX  
685 controls biogenesis of the *Pseudomonas aeruginosa* XcpT-containing pseudopilus. *J*  
686 *Biol Chem* **280**: 31378-31389.

687 Francetic, O., N. Buddelmeijer, S. Lewenza, C. A. Kumamoto and A. P. Pugsley, (2007)  
688 Signal recognition particle-dependent inner membrane targeting of the PulG  
689 pseudopilin component of a type II secretion system. *J Bacteriol* **189**: 1783-1793.

690 Gault, G., M. Ferber, S. Machata, A.-F. Imhaus, C. Malosse, A. A. Charles-Orszag, *et al.*,  
691 (2015) *Neisseria meningitidis* type IV pili composed of sequence invariable pilins are  
692 masked by multisite glycosylation. *PLoS pathogens* **11**: e1005162.

693 Gault, J., C. Malosse, S. Machata, C. Millien, I. Podglajen, M. Ploy, *et al.*, (2014) Complete  
694 posttranslational modification mapping of pathogenic *Neisseria meningitidis* pilins  
695 requires top-down mass spectrometry. *Proteomics* **14**: 1141-1151.

696 Georgiadou, M., M. Castagnini, G. Karimova, D. Ladant and V. Pelicic, (2012) Large-scale  
697 study of the interactions between proteins involved in type IV pilus biology in  
698 *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly.  
699 *Mol Microbiol.* **84**: 857-873.

700 Gold, V.A.M., R. Salzer, B. Averhoff and W. Kühlbrandt. (2015) Structure of a type IV pilus  
701 machinery in the open and closed state. *eLife* **10**, 7554/eLife.07380.

702 Gray, M. D., M. Bagdasarian, W. G. Hol and M. Sandkvist, (2011) In vivo cross-linking of  
703 EpsG to EpsL suggests a role for EpsL as an ATPase-pseudopilin coupling protein in  
704 the type II secretion system of *Vibrio cholerae*. *Mol. Microbiol.* **79**: 786-798.

705 Hu, N.-T., Leu, W.-M., Lee, M.-S., Che, S.-C., Chen, A., Song, Y.-L. and Che, L.-Y., (2002)  
706 XpsG, the major pseudopilin in *Xanthomonas campestris*, pv. *campestris* forms a  
707 pilus-like structure between cytoplasmic and the outer membranes. *Biochem J.* **365**:  
708 205-211.

709 Johnson, T. L., M. E. Scott and M. Sandkvist, (2007) Mapping critical interactive sites within  
710 the periplasmic domain of the *Vibrio cholerae* type II secretion protein EpsM. *J*  
711 *Bacteriol* **189**: 9082-9089.



712 Karimova, G., J. Pidoux, A. Ullmann and D. Ladant, (1998) A bacterial two-hybrid system  
713 based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. USA* **95**:  
714 5752-5756.

715 Karuppiah, V., D. Hassan, M. Saleem and J. P. Derrick, (2010) Structure and oligomerization  
716 of the PilC type IV pilus biogenesis protein from *Thermus thermophilus*. *Proteins* **78**:  
717 2049-2057.

718 Kohler, R., K. Schafer, S. Muller, G. Vignon, K. Diederichs, A. Philippsen, *et al.*, (2004)  
719 Structure and assembly of the pseudopilin PulG. *Molecular microbiology* **54**: 647-664.

720 Korotkov, K., M. Sandkvist and W. Hol, (2012) The type II secretion system: biogenesis,  
721 molecular architecture and mechanism. *Nat Rev Microbiol* **10**: 336-351.

722 Korotkov, K. V. and W. G. Hol, (2008) Structure of the GspK-GspI-GspJ complex from the  
723 enterotoxigenic *Escherichia coli* type 2 secretion system. *Nat Struct Mol Biol.* **15**: 462-  
724 468.

725 Korotkov, K. V., T. L. Johnson, M. G. Jobling, J. Pruneda, E. Pardon, A. Heroux, *et al.*,  
726 (2011) Structural and functional studies on the interaction of GspC and GspD in the  
727 type II secretion system. *PLoS pathogens* **7**: e1002228.

728 Lallemand, M., F. Login, N. Guschinskaya, C. Pineau, G. Effantin, X. Robert, *et al.*, (2013)  
729 Dynamic Interplay between the periplasmic and transmembrane domains of GspL  
730 and GspM in the type II secretion system. *PLoS One* **8**: e79562.

731 Lybarger, S.R., T.L. Johnson, M.D. Gray, A.E. Sikora and M. Sandkvist. (2009) Docking and  
732 assembly of the type II secretion complex of *Vibrio cholerae*. *J Bacteriol.* **191**:3149-  
733 3161.

734 McLaughlin, L., R. Haft and K. Forest, (2012) Structural insights into the Type II secretion  
735 nanomachine. *Cu Op Struct Biol* **22**: 208-216.

736 Miller, J. H., (1972) *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold  
737 Spring Harbor, New York.

738 Nguyen, Y., S. Sugiman-Marangos, H. Harvey, S. D. Bell, C. L. Charlton, M. S. Junop, *et al.*,  
739 (2015) *Pseudomonas aeruginosa* minor pilins prime type IVa pilus assembly and  
740 promote surface display of the PilY1 adhesin. *J Biol Chem* **290**: 601-611.

741 Nivaskumar, M., G. Bouvier, M. Campos, N. Nadeau, X. Yu, E. Egelman, *et al.*, (2014)  
742 Distinct docking and stabilization steps of the pseudopilus conformational transition  
743 path suggest rotational assembly of type IV pilus-like fibers. *Structure* **22**: 685–696.

744 Nivaskumar, M. and O. Francetic, (2014) Type II secretion system: a magic beanstalk or a  
745 protein escalator. *BBA* **1843**: 1568-1577.

746 Nunn, D. and S. Lory, (1991) Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin  
747 leader peptidase. *Proc Natl Acad Sci U S A* **88**: 3281-3285.

748 Parge, H.E., K.T. Forest, M.J. Hickey, D.A. Christensen, E.D. Getzoff and J.A. Tainer, (1995)  
749 Structure of the fibre-forming protein pili at 2.6 Å resolution. *Nature* **378**: 32-38.

750 Pasloske, B. L. and W. Paranchych, (1988) The expression of mutant pilins in *Pseudomonas*  
751 *aeruginosa*: fifth position glutamate affects pilin methylation. *Mol Microbiol.* **2**: 489-  
752 495.

753 Pasloske, B. L., D. G. Scraba and W. Paranchych, (1989) Assembly of mutant pilins in  
754 *Pseudomonas aeruginosa*: formation of pili composed of heterologous subunits. *J*  
755 *Bacteriol* **171**: 2142-2147.

756 Possot, O., G. Vignon, N. Bomchil, F. Ebel and A. P. Pugsley, (2000) Multiple interactions  
757 between pullulanase secretion components involved in stabilization and cytoplasmic  
758 membrane association of PulE. *J Bacteriol* **182**: 2142-2152.

759 Pugsley, A., (1993) Processing and methylation of PulG, a pilin-like component of the  
760 general secretory pathway of *Klebsiella oxytoca*. *Mol Microbiol.* **9**: 295-308.

761 Py, B., F. Loiseau and F. Barras, (2001) An inner membrane platform in the type II secretion  
762 machinery of Gram-negative bacteria. *EMBO Rep.* **2**: 244–248.

763 Sandkvist, M., M. Bagdasarian, S. P. Howard and V. J. DiRita, (1995) Interaction between  
764 the autokinase EpsE and EpsL in the cytoplasmic membrane is required for  
765 extracellular secretion in *Vibrio cholerae*. *The EMBO journal* **14**: 1664-1673.

- 766 Sauvonnet, N., G. Vignon, A. P. Pugsley and P. Gounon, (2000) Pilus formation and protein  
767 secretion by the same machinery in *Escherichia coli*. *EMBO. J.* **19**: 2221-2228.
- 768 Shahapure, R., R. Driessen, M. Haurat, S. Albers and R. Dame, (2014) The archaellum: a  
769 rotating type IV pilus. *Molecular microbiology* **91**: 716-723.
- 770 Strom, M. and S. Lory, (1991) Amino acid substitutions in pilin of *Pseudomonas aeruginosa*.  
771 *J Biol Chem* **266**: 1656-1664.
- 772 Strom, M., D. Nunn and S. Lory, (1993) A single bifunctional enzyme, Pild, catalyzes  
773 cleavage and N-methylation of proteins belonging to the type IV pilin family. *Proc Natl*  
774 *Acad Sci U S A.* **90**: 2404-2408.
- 775 Takhar, H., K. Kemp, M. Kim, P. L. Howell and L. L. Burrows, (2013) The platform protein is  
776 essential for Type IV Pilus Biogenesis. *J Biol Chem* **288**: 9721-9728.
- 777 Vignon, G., R. Köhler, E. Larquet, S. Giroux, M. C. Prévost, P. Roux, *et al.*, (2003) Type IV-  
778 like pili formed by the type II secretion: specificity, composition, bundling, polar  
779 localization, and surface presentation of peptides. *J Bacteriol.* **185**: 3416-3428.
- 780 Voulhoux, R., G. Ball, B. Ize, M. V. Vasil, A. Lazdunski, L.-F. Wu, *et al.*, (2001) Involvement  
781 of the twin arginine translocation system in protein secretion via the type II pathway.  
782 *The EMBO journal* **20**: 6735-6741.
- 783 Yanez , M., K. Korotkov , J. Abendroth and W. G. J. Hol, (2008) The Crystal Structure of a  
784 Binary Complex of two Pseudopilins: EpsI and EpsJ from the Type 2 Secretion  
785 System of *Vibrio vulnificus*. *J. Mol. Biol.* **375**: 471–486.
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<b>Name</b>	<b>Origin/resistance</b>	<b>Relevant markers</b>	<b>Source/reference</b>
pUT18c	ColE1/Ap <sup>R</sup>	<i>placZ</i> -T18	(Karimova <i>et al.</i> , 1998)
pKT25	p15A/Km <sup>R</sup>	<i>placZ</i> -T25	(Karimova <i>et al.</i> , 1998)
pUT18c-Zip	ColE1/Ap <sup>R</sup>	GCN4 (Leu zipper region) fused to T18	(Karimova <i>et al.</i> , 1998)
pKT25-Zip	p15A/Km <sup>R</sup>	GCN4 (Leu zipper region) fused to T25	(Karimova <i>et al.</i> , 1998)
pSU18	p15A/Cm <sup>R</sup>	<i>placZ</i> , <i>lacZ'</i>	(Bartolomé <i>et al.</i> , 1991)
pCHAP1205	p15A/Cm <sup>R</sup>	pSU18 <i>pulG</i>	(Possot <i>et al.</i> , 2000)
pCHAP1329	p15A/Cm <sup>R</sup>	pSU18 <i>pulJ</i>	(Possot <i>et al.</i> , 2000)
pCHAP1331	p15A/Cm <sup>R</sup>	pSU18 <i>pulH</i>	(Possot <i>et al.</i> , 2000)
pCHAP1351	p15A/Cm <sup>R</sup>	pSU18 <i>pull</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP1362	p15A/Cm <sup>R</sup>	pSU18 <i>pulG</i> -His <sub>6</sub>	(Kohler <i>et al.</i> , 2004)
pCHAP2393	ColE1/Ap <sup>R</sup>	pUC18 <i>pulM</i>	(Possot <i>et al.</i> , 2000)
pCHAP6117	p15A/Cm <sup>R</sup>	pSU18 <i>pulJ</i> <sup>E5A</sup>	This study
pCHAP7330	ColE1/Ap <sup>R</sup>	pUT18c <i>pulG</i>	(Nivaskumar <i>et al.</i> , 2014)
pCHAP7332	p15A/Km <sup>R</sup>	pKT25 <i>pulG</i>	(Nivaskumar <i>et al.</i> , 2014)
pCHAP7785	p15A/Cm <sup>R</sup>	pSU18 <i>pulG</i> <sup>E5A</sup> -His <sub>6</sub>	This study
pCHAP7790	p15A/Cm <sup>R</sup>	pSU18 <i>pulG</i> <sup>E5A</sup>	This study
pCHAP8113	ColE1/Ap <sup>R</sup>	pUT18c <i>pulC</i>	This study
pCHAP8119	p15A/Km <sup>R</sup>	pKT25 <i>pulC</i>	This study
pCHAP8154	ColE1/Ap <sup>R</sup>	pUT18c <i>pulM</i>	This study
pCHAP8155	p15A/Km <sup>R</sup>	pKT25 <i>pulM</i>	This study
pCHAP8184	ColE1/Ap <sup>R</sup>	pCHAP8185 $\Delta$ <i>pulG</i>	(Campos <i>et al.</i> , 2010)
pCHAP8185	ColE1/Ap <sup>R</sup>	<i>pulS</i> , <i>pulA</i> <sub>NA</sub> <i>pulB</i> , <i>pulCDEFGHIJKLMNO</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8201	ColE1/Ap <sup>R</sup>	pCHAP8185 <i>pulH::kan</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8209	ColE1/Ap <sup>R</sup>	pCHAP8185 <i>pulJ::kan</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8212	ColE1/Ap <sup>R</sup>	pCHAP8185 <i>pulK::kan</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8218	ColE1/Ap <sup>R</sup>	pCHAP8185 <i>pull::kan</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8245	ColE1/Ap <sup>R</sup>	pUT18c- <i>pull</i>	(Cisneros <i>et al.</i> , 2012)

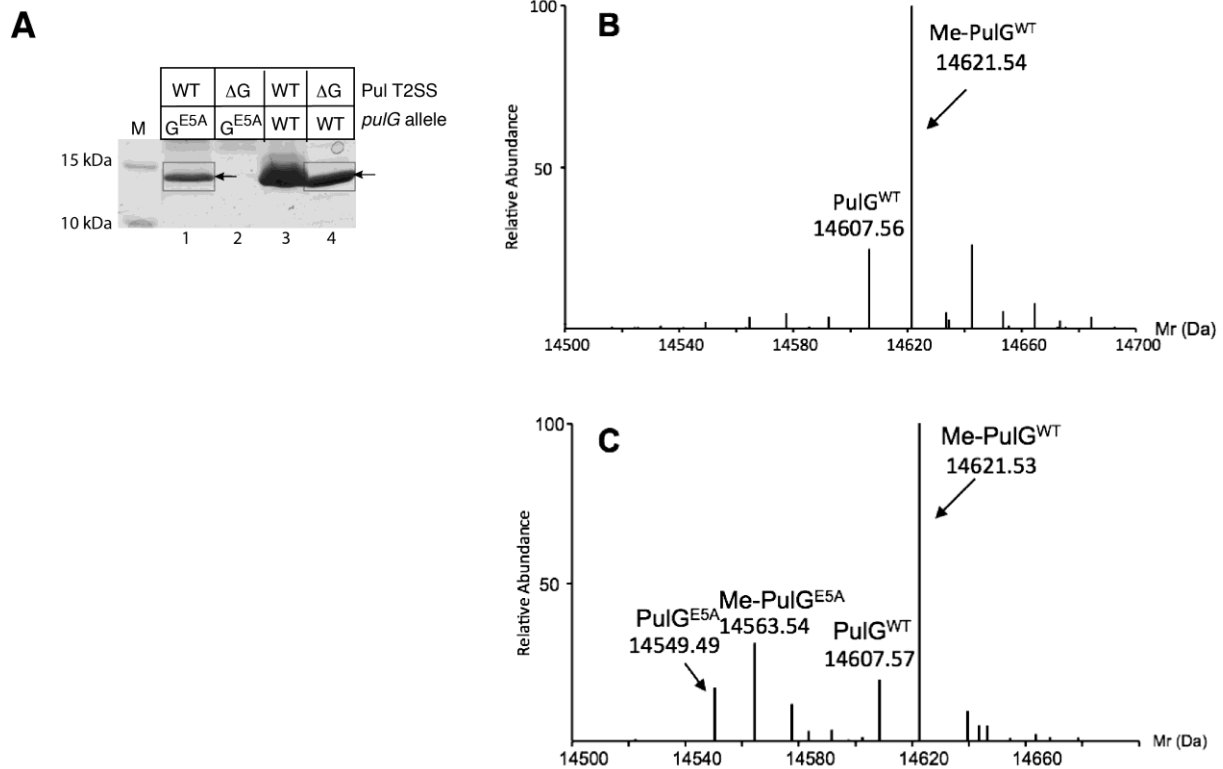
pCHAP8246	ColE1/Ap <sup>R</sup>	pUT18c <i>pulJ</i>	This study
pCHAP8247	ColE1/Ap <sup>R</sup>	pUT18c <i>pulK</i>	This study
pCHAP8248	p15A/Km <sup>R</sup>	pKT25 <i>pulI</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8249	p15A/Km <sup>R</sup>	pKT25 <i>pulJ</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8250	p15A/Km <sup>R</sup>	pKT25 <i>pulK</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8256	ColE1/Ap <sup>R</sup>	pUT18c <i>pulH</i>	This study
pCHAP8257	p15A/Km <sup>R</sup>	pKT25 <i>pulH</i>	(Cisneros <i>et al.</i> , 2012)
pCHAP8364	ColE1/Ap <sup>R</sup>	pUT18c <i>pulF</i>	This study
pCHAP8365	p15A/Km <sup>R</sup>	pKT25 <i>pulF</i>	This study
pCHAP8418	p15A/Cm <sup>R</sup>	pSU18 <i>pulH</i> <sup>E5A</sup>	This study
pCHAP8420	p15A/Km <sup>R</sup>	pKT25 <i>pulG</i> <sup>E5A</sup>	(Nivaskumar <i>et al.</i> , 2014)
pCHAP8434	p15A/Km <sup>R</sup>	pKT25 <i>pulH</i> <sup>E5A</sup>	This study
pCHAP8446	ColE1/Ap <sup>R</sup>	pUT18c <i>pulH</i> <sup>E5A</sup>	This study
pCHAP8472	ColE1/Ap <sup>R</sup>	pUT18c <i>pulL</i>	This study
pCHAP8484	p15A/Km <sup>R</sup>	pKT25 <i>pulL</i>	This study
pCHAP8568	p15A/Cm <sup>R</sup>	pSU18 <i>pulG</i>	This study
pCHAP8639	p15A/Cm <sup>R</sup>	pSU18 <i>pulK</i> <sup>M5E</sup>	This study
pCHAP8670	ColE1/Ap <sup>R</sup>	pUT18c <i>pulG</i> <sup>E5A</sup>	(Nivaskumar <i>et al.</i> , 2014)

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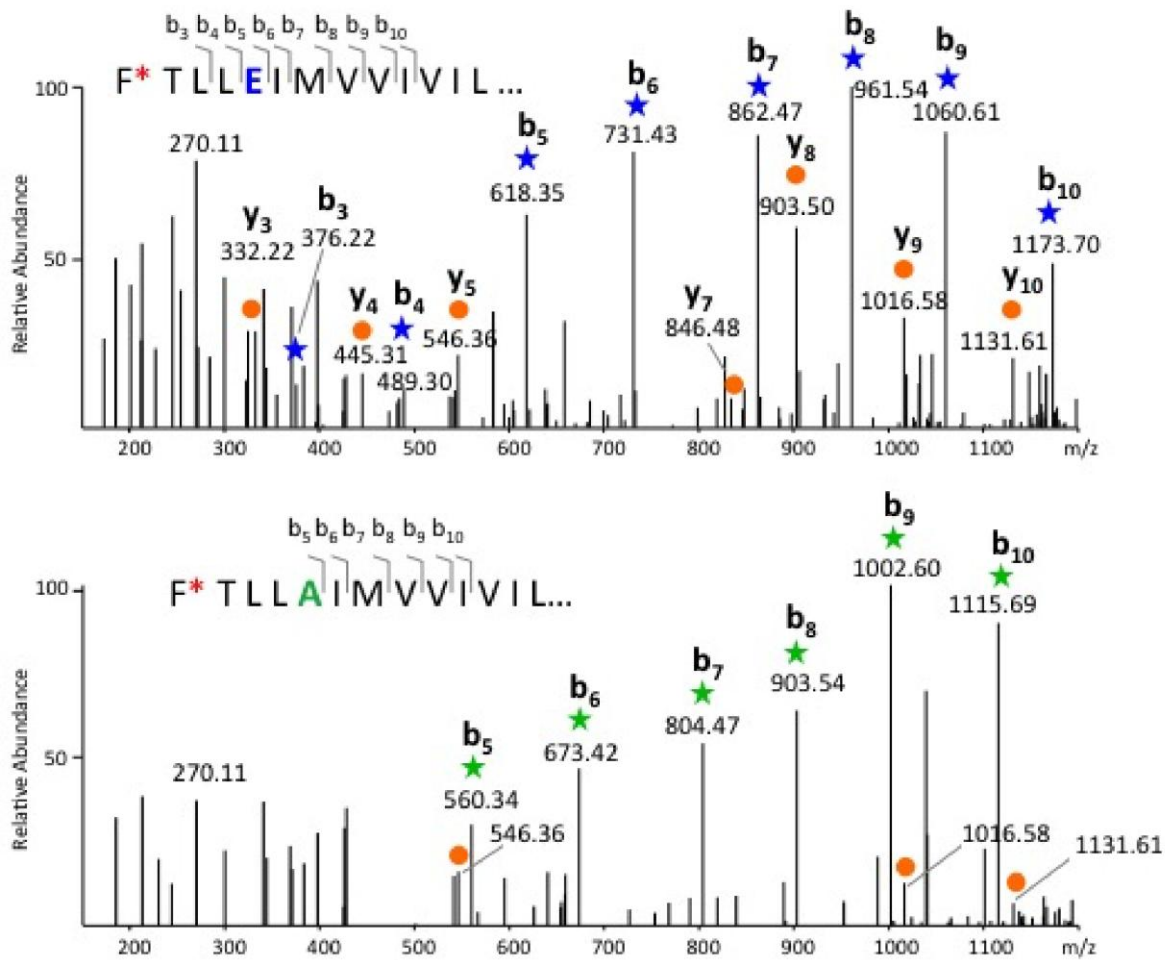
## Figure Legends



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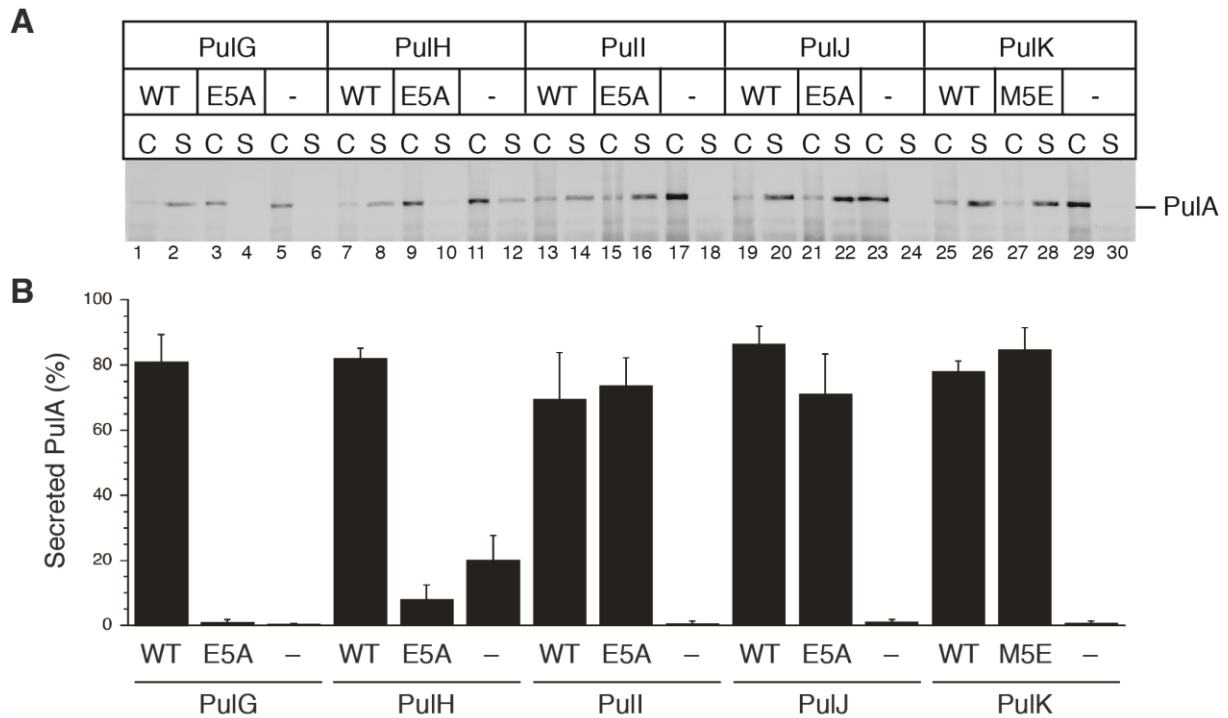
796 **Figure 1.** PulG<sup>E5A</sup> is N-methylated and co-assembled into pili with PulG<sup>WT</sup>. **A.** SDS-PAGE  
797 and Coomassie Blue stained sheared fractions from equivalent amounts of bacteria of strain  
798 PAP7460 carrying two compatible plasmids (Table 1), as indicated: lane 1, pCHAP8185  
799 (encoding the complete Pul T2SS) and pCHAP7790 (encoding PulG<sup>E5A</sup>); lane 2, pCHAP8184  
800 (encoding Pul T2SS lacking PulG) and pCHAP7790; lane 3, pCHAP8185 and pCHAP1205  
801 (encoding PulG<sup>WT</sup>); and lane 4, pCHAP8184 and pCHAP1205. Only the relevant portions of  
802 the gel are shown, with the molecular mass markers indicated on the left. The expected  
803 molecular mass of PulG is around 14.6 kDa. The squares and arrowheads indicate samples  
804 that were further analysed by mass spectrometry. **B.** Deconvoluted high-resolution mass  
805 spectrum (in Mr) obtained for the sample in lane 4 (PAP7460 carrying *pulG*<sup>WT</sup> allele)  
806 indicating the presence of a minor peak at 14607.56 Da corresponding to PulG<sup>WT</sup> and a  
807 major peak at 14621.54 Da corresponding to methylated PulG<sup>WT</sup>; **C.** Deconvoluted high  
808 resolution mass spectrum (in Mr) obtained for the sample in lane 1 (PulG<sup>WT</sup> + PulG<sup>E5A</sup>)  
809 indicating the presence of supplementary peaks corresponding to PulG<sup>E5A</sup> (14549.49 Da)  
810 and methylated PulG<sup>E5A</sup> (14563.53 Da).

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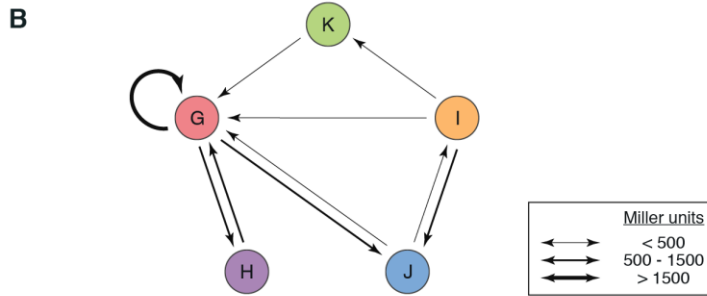
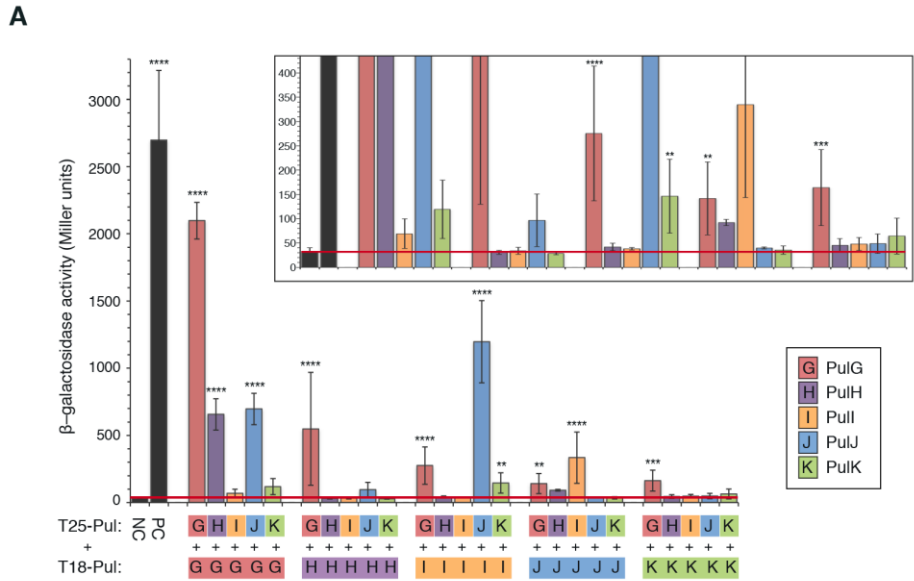


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**Figure 2.** High-energy Collision Dissociation (HCD) analysis of methylated PulG<sup>WT</sup> and PulG<sup>E5A</sup> variants co-assembled into pili. HCD fragmentation spectra of the (14+) ion of methylated PulG<sup>WT</sup> at 1046.05 (top) and methylated PulG<sup>E5A</sup> at 1041.83 (bottom). Methylation of the N-terminal residue is indicated with an asterisk. The series of N-terminal b-type ions obtained in both cases and marked with a star unambiguously delineates the presence of an E at the 5<sup>th</sup> position in PulG<sup>WT</sup> and an A in PulG<sup>E5A</sup>. C-terminal y-type ions are depicted with a circle.

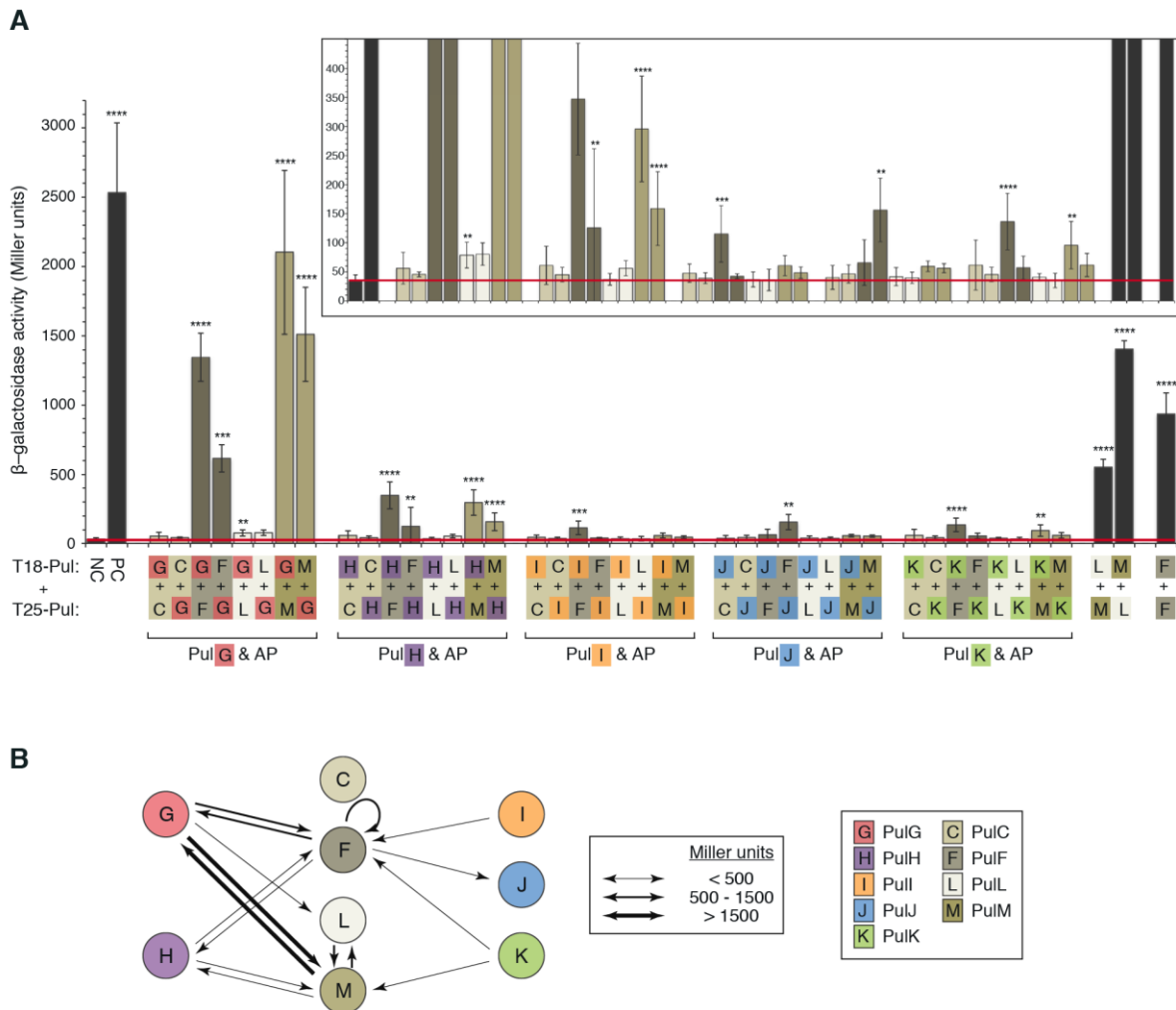


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823 **Figure 3.** Effect of 5<sup>th</sup> residue substitutions on pseudopilin function. Pullulanase (PulA)  
824 secretion was assayed in *E. coli* strain PAP5207 containing pCHAP8185 derivatives with  
825 single deletions of pseudopilin genes was complemented with pSU18 plasmid (-) or its  
826 derivatives expressing the wild type (WT) or the mutant (E5A or M5E) allele of the missing  
827 pseudopilin gene as indicated above the lanes. **A.** A representative of three independent  
828 experiments is shown. Equivalent of 0.05 OD<sub>600nm</sub> of cell (C) or supernatant (S) fractions  
829 were analysed on 9% Tris-Tricin SDS-PAGE and detected using anti-PulA antibodies. **B.**  
830 Quantification of the fraction of secreted PulA in the three independent experiments shown in  
831 panel A and in Figure S1. Bar graphs represent the mean values and the error bars indicate  
832 standard deviation.  
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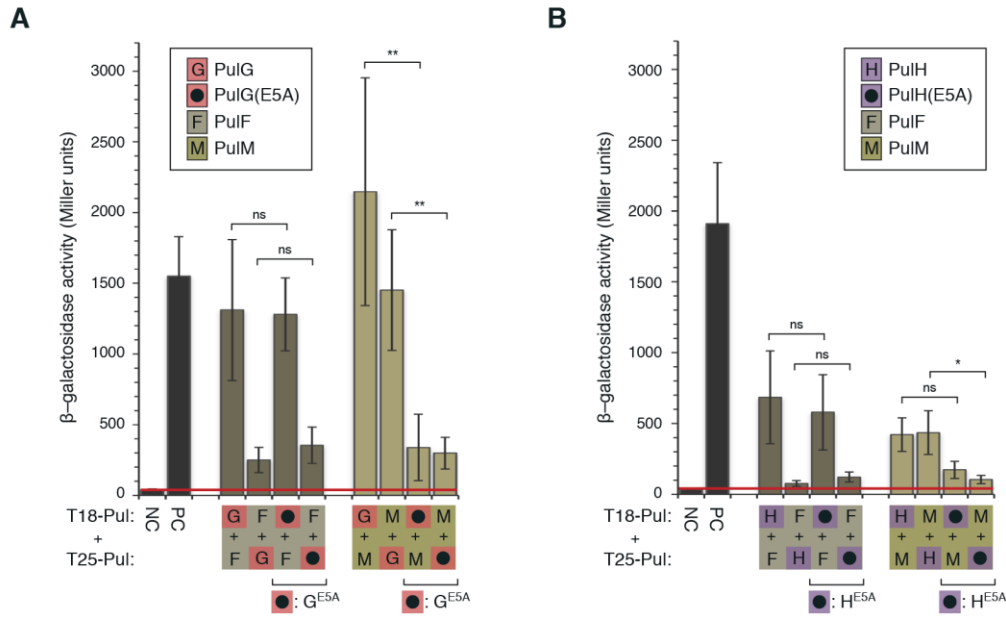
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836 **Figure 4.** Pseudopilin interaction network determined by the bacterial two-hybrid analysis. **A.**  
837 Beta-galactosidase activities of DHT1 bacteria co-producing indicated T18 and T25 chimera  
838 determined as described in Experimental Procedures and expressed in Miller units. NC,  
839 activity of bacteria producing T18 and T25 as negative control; PC, activity of positive control  
840 strain producing T18-Zip and T25-Zip chimera. Each bar represents mean beta-  
841 galactosidase activity value from at least 3 independent colonies obtained by co-  
842 transformation of pKT25 and pUT18c derivatives encoding pseudopilin chimera (T25 or T18  
843 fused to the N-terminus of mature pseudopilins) indicated by the colour code in the inset:  
844 PulG, red; PulH, purple; PulI, orange; PulJ, blue; and PulK, green. The colours of the bars  
845 correspond to the T25-fused proteins. The red horizontal line indicates the background mean  
846 beta-galactosidase activity measured in the negative control. Error bars indicate standard  
847 deviation. Statistical significance relative to the negative controls is indicated above graphs  
848 as follows: \*\*\*\* =  $p < 0.001$ ; \*\*\* =  $P < 0.01$ ; \* =  $P < 0.1$ ; ns = non-significant. The inset above the  
849 main graph shows the part of the same graph with the scale of beta-galactosidase activities  
850 expanded in the low range (from 0 to 500 Miller units). **B.** The results are summarised  
851 schematically in a pentagram depicting pseudopilin arrangement in a putative right-handed  
852 helical complex. Arrows are oriented from T18- to T25- chimera and their line thickness  
853 indicates the strengths of significant interactions, in the range of beta-galactosidase activities  
854 (in Miller units) defined as shown in the legend: weak ( $50 > \text{mean} > 500$ ), strong  
855 ( $500 > \text{mean} > 1500$ ) and very strong ( $\text{mean} > 1500$ ).





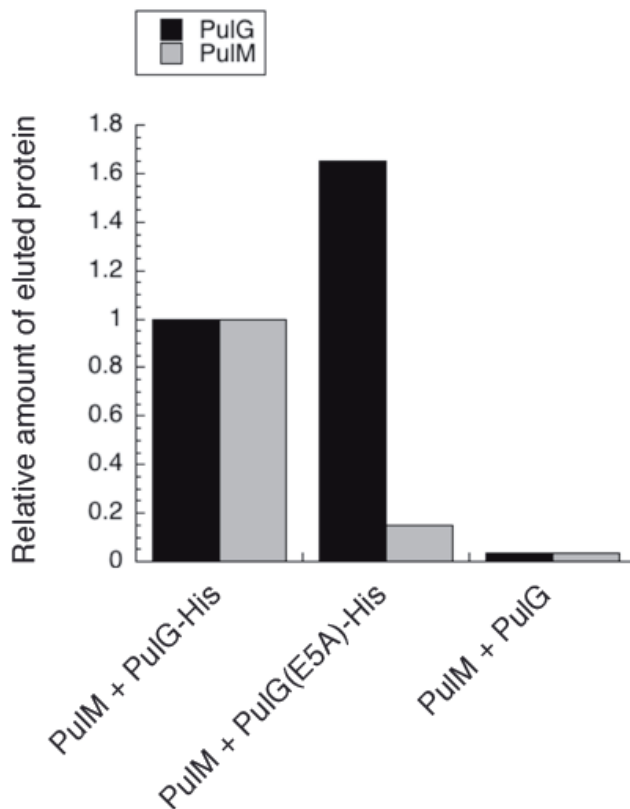
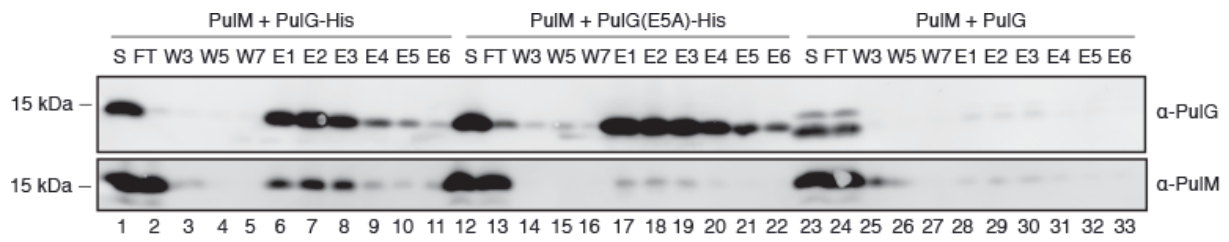
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**Figure 5.** Interactions of pseudopilins with assembly platform components identified using bacterial two-hybrid analysis. **A.** Beta-galactosidase activities of DHT1 bacteria producing indicated T18-Pul or T25-Pul hybrids as bait in the presence of AP component chimera. The Pul components analysed are indicated by the single letter and colour code in the inset. NC, negative control strain co-producing T18 and T25 fragments; PC, positive control co-producing T18-Zip and T25-Zip chimera. The bars represent mean values from at least 3 independent colonies resulting from co-transformation of strain DHT1 with indicated pKT25 and pUT18c derivatives. The colours of the bars correspond to the AP proteins tested. The red line indicates the background beta-galactosidase activity measured in the NC. Error bars indicate standard deviation. Statistically significant mean values relative to the negative control (Experimental Procedures and Supplementary Dataset 1) are indicated above bars. The inset above the main graph shows the part of the same graph with the expanded scale of beta-galactosidase activities in the low range (from 0 to 500 Miller units). **B.** Summary of the interaction data, with the arrows oriented from T18- to T25- chimera and with line thickness corresponding to arbitrary range of interaction strengths indicated in the legend, as in Figure 4.



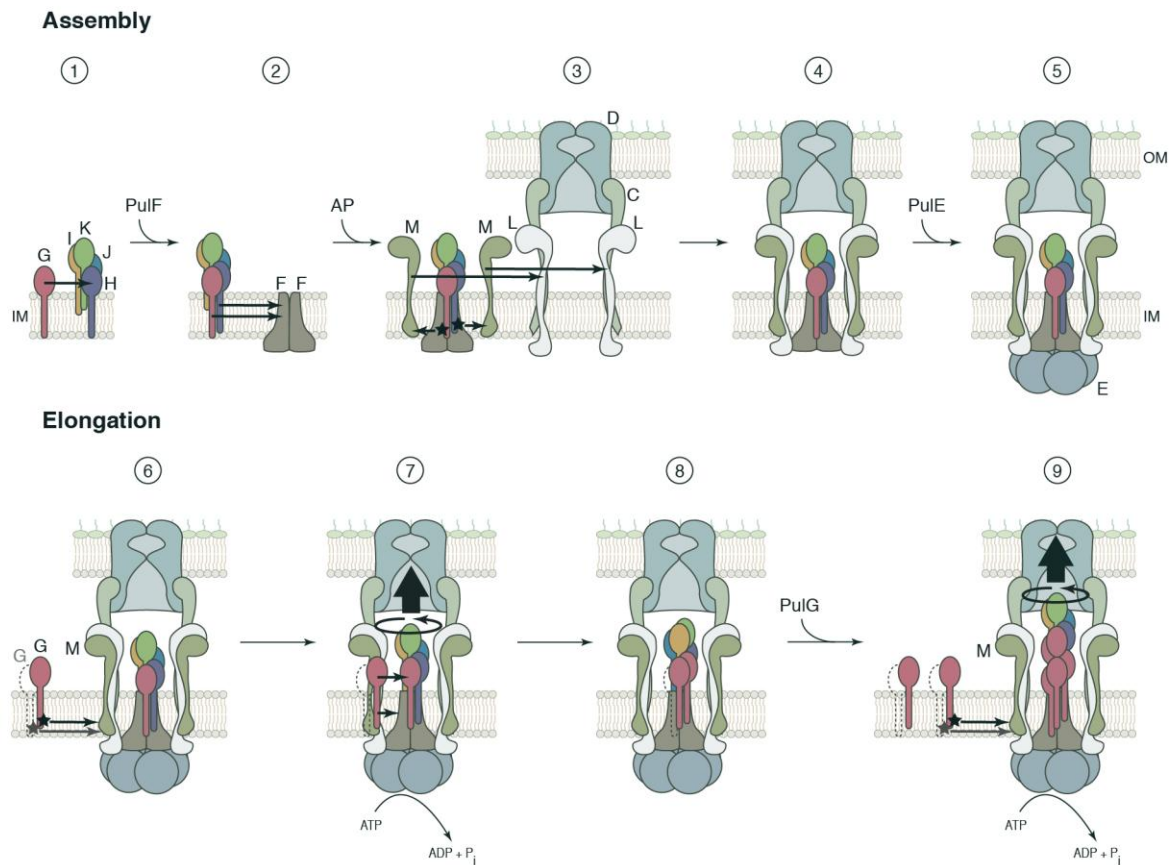
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**Figure 6.** The effect of E5A substitution on PulG and PulH interactions with PulF and PulM. **A.** Bacterial two-hybrid analysis of PulG and PulG<sup>E5A</sup> interactions with PulF and PulM. **B.** Bacterial two-hybrid analysis of PulH and PulH<sup>E5A</sup> interactions with PulF and PulM. Each bar represents the mean value from at least 3 independent colonies obtained by transformation of pKT25 and pUT18c derivatives containing indicated inserts. The colours of the bars correspond to the AP proteins. Error bars indicate standard deviation. NC, negative control and PC, positive control as indicated in the legend of Figure 4. The red line indicates the background beta-galactosidase activity of the NC. Statistical significance of multiple pairwise comparisons is indicated by stars, as in Figure 4.



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**Figure 7.** Co-purification of PulM with PulG-His<sub>6</sub>, PulG<sup>E5A</sup>-His<sub>6</sub> and PulG. Triton X-100 soluble extracts of total membranes from *E. coli* strains PAP7460 producing PulM and the indicated PulG variants were subject to affinity chromatography on Ni-NTA matrix (Experimental Procedures). **A.** SDS-PAGE and immuno-detection of PulG and PulM in fractions of the co-purification. S, solubilised membrane fractions; FT, flow-through; W1, W3 and W7, wash fractions; E1-E6, elution fractions. **B.** Quantification of the relative amount of eluted PulG and PulM relative to wild type levels, according to the densitometric analysis of the bands in (A).



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**Figure 8.** The working model of pseudopilus assembly and elongation. Assembly (top panel): Mature major pseudopilin PulG interacts with the quaternary complex composed of the priming PulJ-PulI-PulK trimer bound to PulH (step 1). Transmembrane regions of PulG and PulH drive the association of the pentameric *proto-pseudopilus* to a PulF dimer (2). PulM binds to this complex *via* the specific interactions of PulM with E5 residues of PulG and PulH, depicted as stars (3). The PulF-pseudopilin-PulM complex associates with PulL within the pre-assembled secretin PulD in the OM bound to the IM protein PulC. The initiation complex (4) recruits hexameric ATPase PulE *via* the cytoplasmic regions of PulL and possibly *via* PulF, thus resulting in a complete, functional T2S machine (5). Elongation (bottom panel): Fibre elongation begins with the recruitment of PulG subunits, likely in the form of dimers, by PulM (6). As in step 3, this recruitment requires a direct contact between the E5 residue of PulG and PulM (6). PulG enters the assembly platform, docks to PulF *via* its TM segments and associates with PulG<sup>P+1</sup> protomer of the *proto-pseudopilus* *via* their globular domains (7), through electrostatic contacts described previously (Nivaskumar *et al.*, 2014). ATP hydrolysis causes conformational changes in PulE that are transmitted to PulF through direct contact, driving the rotation of the proto-fibre (7). The incoming PulG protomer is spooled into the fibre, which results in an overall extension of the pseudopilus (7 and 8). Fibre growth comprises multiple cycles of PulG recruitment, docking and extraction (steps 6 to 9).