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# 1 **Structure and assembly of the Enterohemorrhagic *Escherichia coli* type 4 pilus**

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42 21 Key words 10: type 4 pili, EHEC, NMR, cryo-EM, pilin structure, pilus assembly, molecular  
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44 22 modeling, hemorrhagic coli pilus (HCP)

## 45 46 23 47 48 24 49 50 51 25 **Summary**

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53 26  
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55 27 Bacterial type 4a pili are dynamic surface filaments that promote bacterial adherence, motility  
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57 28 and macromolecular transport. Their genes are highly conserved amongst enterobacteria  
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59 29 and their expression in Enterohemorrhagic *Escherichia coli* (EHEC) promotes adhesion to  
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intestinal epithelia and pro-inflammatory signaling. To define the molecular basis of EHEC pilus assembly, we determined the structure of the periplasmic domain of its major subunit PpdD (PpdDp), a prototype of enterobacterial pilin subfamily containing two disulfide bonds. The structure of PpdDp, determined by NMR, was then docked into the electron density envelope of purified EHEC pili obtained by cryo-electron microscopy (cryo-EM). Cryo-EM reconstruction of EHEC pili at ~8 Å resolution revealed extremely high pilus flexibility correlating with a large extended region of the pilin stem. Systematic mutagenesis, functional and interaction analyses identified charged residues essential for pilus assembly. Structural information on exposed regions and interfaces between EHEC pilins is relevant for vaccine and drug discovery.

## Introduction

Type 4 pili (T4P) are thin filamentous polymers that bacteria use to bind to their substrates, move across surfaces by twitching or gliding, and transport macromolecules in and out of the cell (Berry and Pelicic, 2015). T4P also promote formation of bacterial aggregates and biofilms, and mediate signaling to host cells during infection. The Enterobacteriaceae family of gamma proteobacteria comprises many commensal and environmental species, but also important human, animal and plant pathogens from the genus *Klebsiella*, *Yersinia* or *Dickeya*. Most enterobacteria contain gene clusters encoding pili of the T4a subclass in conserved chromosomal loci (Pelicic, 2008) and share high sequence conservation of major pilin subunits, designated PpdD in *E. coli* (Luna Rico et al., 2019). However, their expression and function have been described only in few species. In the plant pathogen *Erwinia amylovora*, PpdD pili promote biofilm formation and mutants deleted for the *ppdD* operon show reduced virulence (Koczan et al., 2011). In specific nutrient-limiting conditions, PpdD pili are also assembled by enterohemorrhagic *E. coli*

57 (EHEC) (Xicohtencatl-Cortes et al., 2007). EHEC is an important human pathogen causing  
58 outbreaks of severe intestinal infections, with hemolytic uremic syndrome (HUS) as a life-  
59 threatening complication of the disease. Sera of patients recovering from HUS contain  
60 antibodies against PpdD, suggesting that T4P represent one of the virulence factors in the  
61 arsenal of this pathogen (Monteiro et al., 2016).

62 The EHEC pili, also named HCP for *hemorrhagic coli pili*, promote adhesion to  
63 intestinal epithelia in bovines, the main reservoir of this pathogen (Xicohtencatl-Cortes et al.,  
64 2007) and induce pro-inflammatory signaling (Ledesma et al., 2010). Expression of pilin  
65 genes in a laboratory *E. coli* strain HB101 promotes biofilm formation and twitching motility  
66 (Xicohtencatl-Cortes et al., 2009), phenotypes typically associated with T4P dynamics.  
67 Transcriptional studies in *E. coli* K-12 revealed that PpdD pilus assembly genes are co-  
68 regulated with DNA uptake genes *via* Sxy (TfoX) competence activator (Sinha et al., 2009),  
69 suggesting a role of these pili in natural transformation and horizontal gene transfer. PpdD  
70 pilus assembly was achieved in a heterologous type 2 secretion system (T2SS) from  
71 *Klebsiella oxytoca* (Sauvonnet et al., 2000; Cisneros et al., 2012), and more recently in the  
72 reconstituted EHEC pilus assembly system in *E. coli* K-12 (Luna Rico et al., 2019). The  
73 highly similar symmetry parameters of T4aP assembled in these different systems indicate  
74 the key role of major pilins as determinants of pilus structure (Luna Rico et al., 2019).

75 EHEC pili are polymers of the major subunit PpdD, which is a prototype of the  
76 enterobacterial class of pilins, present in many important human, animal and plant pathogens  
77 (Luna Rico et al., 2019). In order to understand the molecular and structural basis of  
78 assembly of these major subunits into a pilus, we determined the solution NMR structure of  
79 the periplasmic domain of the EHEC PpdD (PpdDp). We then combined this structure with  
80 the sub-nanometer resolution cryo-EM reconstruction of EHEC pilus to build an atomic model  
81 of the assembled fiber. In addition, site-directed mutagenesis, functional assays and  
82 interaction studies identified PpdD residues crucial for pilus assembly and stability.

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## 84 **Results**

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2 86 *Solution structure of the periplasmic PpdD domain*  
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4 87 In addition to the well-conserved N-terminal hydrophobic segments ( $\alpha$ 1 helix), PpdD  
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6 88 homologues share similar periplasmic domains stabilized by two disulfide bonds at  
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8 89 conserved positions (de Amorim et al., 2014; Luna Rico et al., 2019). To gain molecular  
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10 90 insight into this group of pilins, we solved the structure of the soluble periplasmic domain of  
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12 91 EHEC PpdD (hereafter designated PpdDp) comprising residues 26 to 140 of the mature  
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14 92 protein. To this end we used solution-state NMR, which is particularly adapted for  
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16 93 determination of structure and dynamics of pilin proteins by providing structural insights into  
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18 94 loops and flexible regions, potentially involved in protein-protein and protein-ligand  
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20 95 interactions. The PpdDp structure is very well defined from 2219 NOE-derived distance, 216  
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22 96 dihedral angle and 45 RDC (residual dipolar coupling) restraints as well as 30 hydrogen  
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24 97 bonds. The details of restraints and structural statistics of the 20 lowest energy conformers  
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26 98 representing the solution structure of PpdDp are summarized in Table 1. The structures  
27  
28 99 show high convergence with a mean pairwise root mean square deviation (RMSD) of 0.55  
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30 100 and 1.06 Å for the backbone and the heavy atoms of ordered regions, respectively. The 3-D  
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32 101 structure of PpdDp displays a canonical alpha-beta pilin fold with two disulfide bonds (Figure  
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34 102 1A,B), largely supported by the dense network of NOE-derived restraints (Figure S1). It is  
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36 103 composed of two  $\alpha$ -helices at both extremities ( $\alpha$ 1C as the C-terminal part of  $\alpha$ 1: N26-H54  
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38 104 and  $\alpha$ 3: D123-F134) that are separated by a long  $\alpha/\beta$  loop and a tilted 4-stranded antiparallel  
39  
40 105  $\beta$  sheet (strands  $\beta$ 1- $\beta$ 2- $\beta$ 3- $\beta$ 4) facing the  $\alpha$ 1C helix. The structure is highly stabilized by two  
41  
42 106 disulfide bridges (Figure 1A,D); one is conserved in type 4 pilins (C118-C130) and connects  
43  
44 107  $\alpha$ 3 with  $\beta$ 4. The second (C50-C60) is characteristic for enterobacterial pilins and connects  
45  
46 108  $\alpha$ 1C to the beginning of the  $\alpha/\beta$  loop leading into a short  $3_{10}$  helix ( $\alpha$ 2: G56-C60). This loop is  
47  
48 109 enriched in His, Gly and Asp residues conserved in the PpdD family.  
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56 110 The  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear NOE data for the backbone confirm an overall well-ordered  
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58 111 structure (Figure S2A). Besides the C-terminal tag, which is highly flexible and without any  
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112 stable interaction with the rest of the protein, two regions with low  $^1\text{H}$ - $^{15}\text{N}$  NOE value are  
113 present, including the  $\beta 3/\beta 4$  (G104-G113) and  $\beta 4/\alpha 3$  (I120-S122) loops. The former includes  
114 the two residues whose signals were not observed in the NMR spectra (D106 and V111)  
115 indicating the presence of intermediate exchange on the chemical shift time scale ( $\mu\text{s}$ - $\text{ms}$ )  
116 between different conformations. Consequently, very few long-range NOEs could be  
117 assigned in the  $\beta 3/\beta 4$  loop, consistent with the high variability of this region in the PpdDp  
118 structure ensemble (Figure S1, S2B,C). Surprisingly, residues of the long  $\alpha/\beta$  loop display  
119  $^1\text{H}$ - $^{15}\text{N}$  NOE values representative of a well-ordered region with low flexibility and slow  
120 internal motions, confirmed by chemical shifts analysis (Figure S2D,E). Numerous short- and  
121 long-range NOE restraints are found within the  $\alpha/\beta$  loop and between the loop and the  $\beta 1$   
122 strand. The loop is anchored at both ends by polar interactions to the extremities of the  $\beta 1$   
123 and  $\beta 2$  strands, while short-range backbone hydrogen bonds stabilize the turns at the N- and  
124 C-terminal parts of the  $\alpha/\beta$  loop (Figure S1C).

### *Structure of the PpdD pilus filament*

127 We determined that T4PS PpdD pili and T2SS PpdD pili have the same morphology and  
128 indistinguishable helical parameters (Luna Rico et al., 2019). We therefore combined the  
129 segments extracted from cryo-EM images of PpdD pili assembled by T4PS and T2SS for  
130 helical reconstruction. The variability in the helical parameters was evident (Figure S3A,B)  
131 from a reference-based classification for sorting the 99,678 segments in terms of variable  
132 twist and rise. The mode of this distribution corresponded to an axial rise of 10.8 Å and twist  
133 of 96°, accounting for ~25% of the whole dataset, and this group was used for the helical  
134 reconstruction. After multiple cycles of IHRSR (Egelman, 2000), the symmetry of this  
135 reconstruction converged to a rise of 11.2 Å and a rotation of ~96° per subunit. The overall  
136 resolution of the reconstruction was ~8 Å according to the half-map FSC criterion (Figure  
137 S3C,D), allowing us to identify the location of the well-separated globular pilin domains  
138 forming the outer shell of the filament (Figure 2A). However, the inner core of the pilus has  
139 less density. At high contour level ( $\sim 5\sigma$ ), small rod-shaped densities are observed along the

140 filament axis, but remain disconnected from the main densities of the globular pilin heads  
1 (Figure 2B). At lower contour level, weaker densities appear, connecting the outer globular  
2 141 heads to the inner rods (Figure 2C). Since the PpdDp NMR structure fitted very nicely into  
3  
4 142 the cryo-EM density at the expected location but did not explain the inner rod-shape  
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6 143 densities, we inferred that they actually correspond to the N-terminal transmembrane  
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8 144 segments (TMS). We thus built an atomic model of the PpdD pilus by docking a homology  
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10 145 model of the helical TMS, connected by an extended linker to the fitted PpdDp structure.  
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12 146 After symmetrization and refinement in real-space, the structure of the PpdDp was  
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14 147 conserved with a C $\alpha$  RMSD of 2.3 Å between the NMR structure and the structure in the  
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16 148 pilus (Figure 2D,E). Upon refinement, most, if not all, structural changes are limited to loops.  
17  
18 149 The largest conformational change is the quasi-rigid displacement of the  $\alpha/\beta$  loop towards  
19  
20 150 the pilin core. In the context of the pilus, the N-terminal part of  $\alpha 1$  ( $\alpha 1N$ ) stops at G11 and is  
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22 151 followed by an unstructured region until around P22, where the  $\alpha 1C$  helix begins (Figure 2D).  
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31 154 The PpdD pilus has a diameter of ~60 Å with somewhat loosely packed pilin subunits  
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33 155 forming a right-handed 1-start helix (Figure 3A). Each PpdD subunit P has contacts with six  
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35 156 neighbors, forming three different interfaces with subunits  $P_{\pm 1}$ ,  $P_{\pm 3}$  and  $P_{\pm 4}$  (Figure 3), with  
36  
37 157 respective buried surface areas of 569 Å<sup>2</sup>, 943 Å<sup>2</sup> and 1529 Å<sup>2</sup>. Potential salt-bridges are  
38  
39 158 present in the  $P-P_{+1}$  and  $P-P_{+3}$  interfaces, while the  $P-P_{+4}$  interface mostly involves  
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41 159 hydrophobic contacts via  $\alpha 1N$  (Figure 3C). The  $\beta 3/\beta 4$  loop, well visible and ordered in the  
42  
43 160 cryo-EM density, folds as an elongated extension of the  $\beta$ -sheet, making inter-subunit  
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45 161 contacts with the D-region and with the N-terminal region at the  $P-P_{+4}$  interface (Figure 3C).  
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51 163 Co-evolutionary analysis performed on the PpdD pilin sequence family provided intra- and  
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53 164 inter-pilin contact predictions that support the atomic model of PpdD pilus (Table S1; Figure  
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55 165 S4).  
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60 167 *PpdD mutagenesis and effects on pilus assembly*  
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168 The mature PpdD, N-terminally processed by the prepilin peptidase has 7 positively  
1 and 13 negatively charged residues. The surface of the EHEC pilus is highly negatively  
2 169 and 13 negatively charged residues. The surface of the EHEC pilus is highly negatively  
3 charged (Figure 4A). To assess the role of electrostatic interactions in assembly and  
4 170 charged (Figure 4A). To assess the role of electrostatic interactions in assembly and  
5 stabilization of PpdD pili, we introduced charged residue substitutions that create  
6 171 stabilization of PpdD pili, we introduced charged residue substitutions that create  
7 electrostatic repulsion between PpdD subunits or between PpdD and its assembly  
8 172 electrostatic repulsion between PpdD subunits or between PpdD and its assembly  
9 components. We examined pilus assembly in an *E. coli* strain producing the *K. oxytoca* T2SS  
10 173 components. We examined pilus assembly in an *E. coli* strain producing the *K. oxytoca* T2SS  
11 but lacking its cognate major pseudopilin PulG (Figure 4D,E). The mean piliation efficiency,  
12 174 but lacking its cognate major pseudopilin PulG (Figure 4D,E). The mean piliation efficiency,  
13 expressed as a fraction of total PpdD detected on the bacterial surface, was determined for  
14 175 expressed as a fraction of total PpdD detected on the bacterial surface, was determined for  
15 each of these variants from four independent experiments. About 50% of native PpdD  
16 176 each of these variants from four independent experiments. About 50% of native PpdD  
17 (PpdD<sup>WT</sup>) was assembled into pili in this system, a fraction comparable to the assembly  
18 177 (PpdD<sup>WT</sup>) was assembled into pili in this system, a fraction comparable to the assembly  
19 efficiency for the cognate T2SS substrate, the major pseudopilin PulG assembled into pili  
20 178 efficiency for the cognate T2SS substrate, the major pseudopilin PulG assembled into pili  
21 under the same conditions (Campos et al., 2010). As expected, piliation was abolished for  
22 179 under the same conditions (Campos et al., 2010). As expected, piliation was abolished for  
23 the PpdD<sup>E5A</sup> variant lacking the conserved E5 residue, as in *P. aeruginosa* (Strom and Lory,  
24 180 the PpdD<sup>E5A</sup> variant lacking the conserved E5 residue, as in *P. aeruginosa* (Strom and Lory,  
25 1991) and *Neisseria gonorrhoeae* (Aas et al., 2007). The E5 residue has been implicated in  
26 181 1991) and *Neisseria gonorrhoeae* (Aas et al., 2007). The E5 residue has been implicated in  
27 an early step of subunit recruitment to the assembly machinery (Nivaskumar et al., 2016), as  
28 182 an early step of subunit recruitment to the assembly machinery (Nivaskumar et al., 2016), as  
29 well as in inter-subunit contacts in assembled pili (Kolappan et al., 2016; Wang et al., 2017).  
30 183 well as in inter-subunit contacts in assembled pili (Kolappan et al., 2016; Wang et al., 2017).  
31 Similar to the behavior of PulG<sup>E5A</sup>, which is arrested in the membrane-embedded  
32 184 Similar to the behavior of PulG<sup>E5A</sup>, which is arrested in the membrane-embedded  
33 preassembly state, (Nivaskumar et al., 2014; Santos-Moreno et al., 2017) cellular PpdD<sup>E5A</sup>  
34 185 preassembly state, (Nivaskumar et al., 2014; Santos-Moreno et al., 2017) cellular PpdD<sup>E5A</sup>  
35 levels were consistently higher compared to PpdD<sup>WT</sup>. On the other hand, several PpdD  
36 186 levels were consistently higher compared to PpdD<sup>WT</sup>. On the other hand, several PpdD  
37 variants showed reduced stability, including PpdD<sup>R29E</sup> and, more dramatically, PpdD<sup>R44D</sup>,  
38 187 variants showed reduced stability, including PpdD<sup>R29E</sup> and, more dramatically, PpdD<sup>R44D</sup>,  
39 consistent with the intra-protomer interaction of R44 with E48 identified by NMR. Charge  
40 188 consistent with the intra-protomer interaction of R44 with E48 identified by NMR. Charge  
41 inversion of residues mapping onto the fiber surface had little effect on pilus assembly  
42 189 inversion of residues mapping onto the fiber surface had little effect on pilus assembly  
43 (Figure 4B,C,D,E). With the exception of the surface-exposed residue K83, all positively  
44 190 (Figure 4B,C,D,E). With the exception of the surface-exposed residue K83, all positively  
45 charged residues were essential for piliation (Figure 4C,D,E). Several charge inversions  
46 191 charged residues were essential for piliation (Figure 4C,D,E). Several charge inversions  
47 localized at the interfaces with other subunits abolished pilus assembly, including K30E,  
48 192 localized at the interfaces with other subunits abolished pilus assembly, including K30E,  
49 E48K, D61R, R74D, R116D and R135E. Nearly all residues in the periplasmic part of the  
50 193 E48K, D61R, R74D, R116D and R135E. Nearly all residues in the periplasmic part of the  
51 alpha helical PpdD stem appear to be very important for piliation (Figure 4D,E).  
52 194 alpha helical PpdD stem appear to be very important for piliation (Figure 4D,E).  
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196           Among the inter-protomer contacts detected in the cryo-EM structure of the EHEC  
197 pilus, R74<sub>P+1</sub> is in close proximity to both D137<sub>P</sub> and D138<sub>P</sub> (Figure 3D). Whereas the charge  
198 inversion of residue R74 (R74E/D) abolished piliation, the double charge inversion D137R-  
199 D138K did not. However, piliation of PpdD<sup>R74E</sup> and PpdD<sup>R74D</sup> variants was restored in the  
200 presence of the D137R-D138K substitution, supporting the proximity of these residues and  
201 their importance at the P-P<sub>+1</sub> interface (Figure 3E). Similarly, in a non-piliated PpdD variant  
202 R135E, pilus assembly could be partially rescued by an additional charge inversion of a non-  
203 essential residue E92<sub>P+1</sub>, facing the R135<sub>P</sub> (Figure 3F). This effect was specific since piliation  
204 was not restored in another double mutant variant, PpdD<sup>D35K-R135E</sup> and it is consistent with the  
205 relative proximity of R135 and E92 in the pilus structure (Figure 3F).

#### 207 *Role of PpdD interactions with fiber assembly factors*

208           Recent functional reconstitution of the EHEC T4P system (T4PS) (Luna Rico et al.,  
209 2019) allowed us to compare assembly of PpdD and its variants *via* the T2SS (Figure 5A, top  
210 panel) with assembly *via* its cognate T4PS machinery (Figure 5A, bottom panel). The  
211 majority of PpdD variants with residue substitutions in the N-terminal stem region were  
212 defective for assembly in both systems. Charge inversions at these positions affected  
213 homotypic interactions with PpdD subunits in the membrane, prior to pilus assembly, as  
214 indicated by the bacterial two-hybrid (BACTH) analysis (Figure 5B). A notable exception was  
215 the PpdD<sup>E5A</sup> variant, whose ability to form dimers was indistinguishable from wild type  
216 (Figure 5B). While fully assembly-defective in T2SS, PpdD<sup>E5A</sup> was still assembled in the  
217 cognate T4PS, albeit less efficiently than native PpdD. Several other variants, including  
218 PpdD<sup>R74D</sup> and PpdD<sup>R116D</sup>, produced pili with T4PS but not with T2SS, indicating that  
219 assembly *via* the cognate system was more robust and less sensitive to mutations.

220           In addition to their effect on docking to the pilus or dimerization of pilins in the  
221 membrane, PpdD residue substitutions might differentially affect its interactions with specific  
222 assembly factors in the two systems. Heterologous assembly of PpdD *via* the T2SS is likely  
223 a result of its ability to interact with several T2SS components, including the assembly

224 protein PulM (Luna Rico et al., 2019). The PulM equivalent HofN is the major PpdD  
225 interacting partner in the EHEC T4PS (Luna Rico et al., 2019). To test this possibility, we  
226 examined the effect of charged residue substitutions in PpdD on its interactions with HofN  
227 and PulM in the BACTH assay (Karimova et al., 1998). Although PpdD<sup>E5A</sup> retained the ability  
228 to interact with the assembly factor HofN, this interaction was significantly reduced compared  
229 to PpdD<sup>WT</sup> (Figure 5C). The charge inversion R74D, which abolished piliation in the T2SS  
230 had a limited effect in T4PS; this differential effect apparently correlated with the ability of  
231 PpdD<sup>R74D</sup> variant to interact with HofN, but not with PulM in the BACTH assay (Figure 5C).  
232 The assembly-defective PpdD<sup>D35K</sup> variant, which interacted very poorly with PpdD<sup>WT</sup> (Figure  
233 5B) was also affected for interaction with the assembly factors HofN and PulM. Another  
234 assembly-defective variant PpdD<sup>D135K</sup> showed reduced interaction with HofN and PulM, while  
235 retaining the ability to interact with PpdD<sup>WT</sup> (Figure 5B,C). Together, these data highlight an  
236 important role of electrostatic contacts in interactions with other protomers and with the inner  
237 membrane assembly factors.

### 239 *Dynamic behavior of different T4aP*

240 We used normal mode analysis to obtain information on the dynamic behavior of  
241 EHEC, *N. meningitidis* (Nm), *N. gonorrhoeae* (Ng) and *P. aeruginosa* strain PAK T4aP. For  
242 each T4aP structure, the first 3 non-trivial modes reveal similar dynamic behavior. Modes 1  
243 and 2 correspond to the bending of the filaments in two orthogonal directions, while  
244 movements along mode 3 represent twisting of the filament around the helical axis (Figure  
245 6). For each of the 3 modes, the EHEC T4P structure displays the largest mean square  
246 fluctuations of the pilin head per subunit, while Nm, Ng and PAK T4P display similar  
247 behaviors of lower fluctuations (Figure 6). As expected, the largest fluctuations are observed  
248 at the extremities of the filaments, and in the central region for the bending modes. In the  
249 twisting mode (mode 3), the fluctuation pattern along the pilus axis oscillates, with a  
250 periodicity of 3 subunits correlating with the left-handed 3-start helix present in all T4aP  
251 structures. This observation may indicate the importance of the P-P<sub>+3</sub> interface in the

252 plasticity of T4aP filaments. Interestingly, the EHEC T4P structure displays a significantly  
1 smaller P-P<sub>+3</sub> interface compared to Nm, Ng and PAK (Figure S5).  
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## 8 256 **Discussion**

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12  
13 258 The fold of the periplasmic domain of EHEC PpdD is very similar to known structures  
14  
15 259 of T4a pilins with RMSD for C $\alpha$  atoms ranging from 2.7 to 3.2 Å (Figures 1 and S8 and Table  
16  
17 S2). Major structural differences are restricted to the characteristic hypervariable regions in  
18 260 T4a pilins: the  $\alpha/\beta$  loop, the  $\beta_3/\beta_4$  loop and the D-region, encompassed by the two cysteines  
19  
20 261 forming the C-terminal disulfide bridge (Figure S6). Variable regions confer different surface  
21  
22 262 shape and charge properties to assembled pili, presumably adapted to their specific binding  
23  
24 263 properties and biological function. The most notable variation of the D region reflects the  
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26 264 selection pressure in the face of the immune system (Cehovin et al., 2010).  
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31 266 Among the pilins, the major pilin PilE from *F. tularensis* (PilE<sub>Ft</sub>) is the closest structural  
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33 267 homologue to PpdDp despite sharing the lowest sequence identity (only 16% for the  
34  
35 268 periplasmic domain) (Table S2), indicating the high sequence variability in the structurally  
36  
37 269 conserved T4a pilins. The PilE<sub>Ft</sub> X-ray structure shows two molecules in the asymmetric unit  
38  
39 270 (Hartung et al., 2011) with secondary structure features quite similar to that of PpdDp (Figure  
40  
41 271 1C). The most pronounced structural difference is an extra strand present in PilE<sub>Ft</sub> in the  $\alpha/\beta$   
42  
43 272 loop involved in the crystallographic dimer interface (Hartung et al., 2011). When the two  
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45 273 molecules of PilE<sub>Ft</sub> in the crystallographic dimer are superimposed (Figure 1C), the slightly  
46  
47 274 different conformation in the  $\beta_3/\beta_4$  loop between the two molecules suggests some flexibility.  
48  
49 275 For PpdDp, the flexibility in this region is evidenced by the lower heteronuclear NOE values  
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51 276 (Figure S2). It was shown recently that a short segment in the  $\alpha/\beta$  loop of the N-terminally  
52  
53 277 truncated PilA pilin of *P. aeruginosa* PA14 is highly dynamic in solution while essential for  
54  
55 278 piliation (Nguyen et al., 2018). Interestingly, increased backbone dynamics of both the  $\alpha/\beta$   
56  
57 279 and  $\beta_3/\beta_4$  loops was also observed in monomeric *P. aeruginosa* K122-4 pilin (Suh et al.,  
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280 2001). In PpdD, a charge inversion at position D106 within the dynamic  $\beta 3/\beta 4$  loop affected  
1 pilus assembly specifically in the T2SS, presumably by altering contacts with another PpdD  
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4 282 protomer (Figure 5) or with an assembly factor.

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6 283 Flexible regions might be involved in overall pilus dynamics and in the switching  
7  
8 284 between assembly and retraction modes, required for twitching motility (Clausen et al.,  
9  
10 285 2009). Identifying all interacting partners of the dynamic loops may shed light on the fine  
11  
12 286 tuning of these modes, which are driven by two strong and antagonistic ATPase motors at  
13  
14 287 the base of the system (McCallum et al., 2017). The IM assembly factors PilN and PilO likely  
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16 288 participate in this modulation, although their precise role and mechanism remain unclear  
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18 289 (Leighton et al., 2015).

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20  
21  
22 290 So far, detailed cryo-EM reconstructions of bacterial T4aP have been achieved for *N.*  
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24 291 *meningitidis* (Kolappan et al., 2016), *N. gonorrhoeae* and *P. aeruginosa* (Wang et al., 2017).  
25  
26 292 In all cases, pilin subunits show a similar axial rise of approximately 10 Å, whereas the twist  
27  
28 293 angle is more variable and reflects specific packing of pilin globular domains. The new cryo-  
29  
30 294 EM reconstruction of EHEC pili shows very similar subunit organization and helical  
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32 295 parameters to that of other T4aP (Figure 7). As observed for the other T4aP and the related  
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34 296 T2SS pseudopilus structures recently solved, the  $\alpha 1$  helix of PpdD is interrupted by a melted  
35  
36 297 and extended region. However, this extended region is longer in PpdD, going from G11 to  
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38 298 P22, whereas in Ng, Nm and PAK pilins and in *K. oxytoca* pseudopilin, it starts at G14, a  
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40 299 residue which is not conserved in PpdD. We note that the interruption of the  $\alpha 1$  helix  
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42 300 correlates well with the T4a pilin-specific sequence-based secondary structure predictions  
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44 301 (Figure S7). This extended region at the  $\alpha 1$  helix seems to be a structural feature of T4aP. It  
45  
46 302 has been proposed that the interruption of the helical part may have a role in the ability of  
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48 303 T4P to extend under force, and relax back to a native state when the force is removed (Wang  
49  
50 304 et al., 2017). This flexibility, while crucial for pili functions, is an impediment for their structure  
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52 305 determination at high resolution. The long, extended region of PpdD can explain the limited  
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54 306 resolution of the pilus structure. The enhanced flexibility of EHEC pilus towards bending and  
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56 307 twisting from normal mode analysis also indicates the crucial role of the P-P<sub>+3</sub> interface in  
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308 pilus rigidity. In EHEC, this reduced interface mainly involves the extended region of the pilin  
1 stem, thus supporting its implication in plasticity of pili.  
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4 310 Interestingly, when mapped onto the PpdD pilus structure, the location of  
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6 311 hypervariable pilin regions forms a triangular junction between neighboring pilins at the  
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8 312 filament surface (Figure 4F,G). Their exposed residues can define the specificity of  
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10 interactions with different partners.  
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12  
13 314 Overall, our mutagenesis and functional analyses show that the majority of charged  
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15 315 PpdD residues required for pilus assembly mapped onto the side of the protein facing the  
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17 316 fiber core, consistent with their involvement in inter-protomer interactions in the polymerized  
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19 317 fiber. In addition, we show that some of these residues affect binding to fiber assembly  
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21 318 factors in the plasma membrane, suggesting their role in early assembly steps. Identifying  
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23 319 and characterizing these steps in dynamic terms should shed new light on substrate  
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25 320 specificity of fiber assembly systems. The complex network of electrostatic interactions that  
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27 321 ensures quality control of pilus assembly and correct pilin docking to the assembly site  
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29 322 requires further structure-function analysis. In the related *Klebsiella* T2SS, binding of the  
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31 323 major pseudopilin PulG to PulM has been implicated in subunit targeting to the assembly  
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33 324 machinery (Nivaskumar et al., 2016; Santos-Moreno et al., 2017). PpdD binding to PulM  
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35 325 probably allows for its assembly *via* the T2SS, whereas the PulM equivalent HofN in the  
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37 326 EHEC T4PS might play a similar role, as suggested by the results of BACTH-based  
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39 327 interaction studies.  
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44 328 EHEC pili have been implicated in colonization of mammalian host epithelia  
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46 329 specifically under starvation conditions and there is evidence for their synthesis *in vivo*  
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48 330 (Xicohtencatl-Cortes et al., 2007). Antibodies against PpdD are produced in patients and  
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50 331 they inhibit pro-inflammatory signaling in a dose-dependent manner (Ledesma et al., 2010).  
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52 332 Therefore, targeting T4P is a possible therapeutic strategy for EHEC infections, which are  
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54 333 life-threatening, and where antibiotic treatment is contraindicated.  
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58 334 In addition, the EHEC pili share common regulatory and structural features with the  
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60 335 *Vibrio* T4aP that are involved in natural competence (Matthey and Blokesch, 2016). In a  
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336 recent elegant study, Ellison and colleagues showed that *Vibrio* T4aP capture DNA *via* their  
1 tip and promote its uptake to the periplasm through pilus retraction (Ellison et al., 2018).  
2 337  
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4 338 Removing several positively charged residues of two minor pilin subunits, presumably  
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6 339 localized at the tip of the pilus, significantly affected DNA binding and transformation  
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8 340 efficiency (Ellison et al., 2018). Although DNA binding has been attributed to major pilins in  
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10 341 some bacteria like *P. aeruginosa* (van Schaik et al., 2005) or *Neisseria* spp. (Craig et al.,  
11 342 2006; Imhaus and Duménil, 2014), direct biochemical evidence supports the role of  
12  
13 343 specialized minor pilin ComP in this binding in *N. meningitidis* (Cehovin et al., 2013).  
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15 344 Consistent with this model, we did not observe any interaction of PpdDp with double  
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17 345 stranded DNA by NMR. Moreover, the highly negatively charged surface of the EHEC pilus  
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19 346 makes its direct interaction with DNA unlikely. PpdD is highly similar to another pilin involved  
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21 347 in natural competence, the *Acinetobacter baumannii* PilA, whose recent structural analysis  
22  
23 348 revealed similar features including two disulfide bridges and a highly negatively charged  
24  
25 349 surface (Ronish et al., 2019). Surface charge affected the capacity of PilA to form bundles  
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27 350 and lead to microcolony formation, presumably through electrostatic repulsion (Ronish et al.,  
28  
29 351 2019). In comparison, PAK, Ng and Nm pili display more mixed surface electrostatics, with  
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31 352 almost continuous positive (PAK) and negative (Ng) patches (Figure S8). These might favor  
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33 353 pilus bundling, but also interactions with specific surfaces and substrates.  
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42 355         Specific nutritional or other signals so far unknown may activate DNA uptake *in vivo*,  
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44 356 as suggested by the capacity of *E. coli* to take up exogenous DNA (Sinha and Redfield,  
45  
46 357 2012). Given the high conservation of PpdD and T4aP systems in enterobacteria, this  
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48 358 capacity might be widespread among commensal strains, and among pathogens of the  
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50 359 ESKAPE group like *Klebsiella*, *Salmonella* or *Yersinia* that represent a particular threat due  
51  
52 360 to their antibiotic resistance. Further studies will be required to determine whether and how  
53  
54 361 T4P in enterobacteria participate in DNA uptake and contribute to exchange of genetic  
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56 362 material in the gut. Detailed structural information will be instrumental to gain a better  
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363 understanding of the mechanisms underlying these processes *in vivo*, and is crucial for drug  
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2 364 and vaccine discovery.

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6 366 **Data availability**

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8 367  
9 368 The PpdDp structure and NMR restraints have been deposited in the Protein Data Bank  
10  
11 369 under the accession code 6GMS. The PpdD pilus structure and cryo-EM map are available  
12  
13 370 from the Protein Data Bank (accession code 6GV9) and Electron Microscopy Data Bank  
14  
15 371 (accession code EMD-0070), respectively.  
16  
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34 381  
35  
36 382 **Author contributions**

37 383  
38 384 Conceived and designed the experiments: BB, ALR, MN, EHE, NIP and OF. Performed the  
39  
40 385 experiments: BB, GCA, ALR, WZ, CJ, OF. Analyzed the data: GCA, ALR, BB, WZ, MN, EHE,  
41  
42 386 NIP and OF. Wrote the manuscript: BB, ALR, WZ, MN, EHE, NIP and OF.  
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46 387  
47 388  
48 389 **Declaration of interest**

49 390  
50 391 The authors declare no competing interests.  
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53 392  
54 393 **References**

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## 51 610 52 611 **Main figure titles and legends**

### 53 612 54 613 **Figure 1. Solution NMR structure of PpdDp.**

55 614  
56 615 **(A)** Backbone representation of the NMR ensemble of PpdDp colored by secondary structure  
57 616 elements. Helices  $\alpha$ 1C,  $\alpha$ 2 and  $\alpha$ 3 are shown in red and the  $\beta$ 1- $\beta$ 2- $\beta$ 3- $\beta$ 4 sheet is shown in

617 cyan. Disulfide bridges are colored in yellow. **(B)** Ribbon representation of the lowest energy  
618 model of the PpdDp NMR ensemble. Characteristic hypervariable regions in T4a major pilins  
619 are colored in B, C and D as follows:  $\alpha/\beta$  loop in green,  $\beta 3/\beta 4$  loop in cyan and D-region in  
620 magenta. Disulfide bridges C50-C60 and C118-C130 are shown as yellow sticks. **(C)**  
621 Superposition of the two molecules in the asymmetric unit of the *Francisella tularensis* Pile  
622 crystallographic structure (PDB 3SOJ). The conformationally variable  $\beta 3/\beta 4$  loop is  
623 highlighted by a dotted circle. **(D)** Sequence alignment of full-length PpdD from EHEC, Pile  
624 pilins from *F. tularensis* (Ft) and *Neisseria gonorrhoeae* (Ng). Positions of helix breaking  
625 residues (G, P) in the N-terminal region are highlighted with red arrows. Cysteine residues  
626 involved in disulfide bridges are connected by blue lines. See also Figures S2, S6 and Table  
627 S2.

### 630 **Figure 2. Cryo-EM reconstruction of the EHEC T4P filament at $\sim 8$ Å resolution and** 631 **PpdD pilin structure fitting.**

632 **(A)** Surface view of the EHEC T4P cryo-EM reconstruction at  $\sim 4.2\sigma$  contour level with the  
633 refined atomic model shown in ribbons (red). The diameter of the pilus is  $\sim 60$ Å. **(B-C)** Cross-  
634 sections of the cryo-EM reconstruction at  $\sim 5\sigma$  (B) and  $\sim 3.8\sigma$  (C) contour levels and with a  
635 single PpdD subunit shown in ribbons. **(D)** Structure of a PpdD pilin subunit in the refined  
636 EHEC T4P structure colored by secondary structures and showing the extended region  
637 between the short  $\alpha 1N$  helix and the PpdDp domain. The positions of helix breaking between  
638 residues G11 and P22 are indicated. **(E)** Superposition of the lowest-energy conformer of  
639 monomeric PpdDp in solution (blue) with the refined pilus structure (red) shown in two  
640 orientations. See also Figure S3.

### 642 **Figure 3. Structure of the EHEC PpdD pilus.**

643 **(A)** Surface representation of the PpdD pilus structure with subunits P,  $P_{+1}$ ,  $P_{+2}$ ,  $P_{+3}$  and  $P_{+4}$   
644 (along the 1-start helix) colored in orange, cyan, green, red and magenta, respectively. **(B)**  
645 Structure of PpdD pilus shown in ribbons where subunits are colored as in (A). The helical  
646 rise of the 1-start helix is 11.2 Å. **(C)** Topological arrangement of neighboring subunits in the  
647 PpdD pilus structure, colored as in (A). The positions of charged residues involved in inter-  
648 subunit interactions are labeled with colored outline. Residues making hydrophobic contacts  
649 between subunits P and  $P_{-3}/P_{-4}$  are labelled in black. **(D)** Close-up view of the interface  
650 between protomer P and  $P_{+1}$  showing the side-chains of D137/D138 and R74 making  
651 potential salt-bridges in the PpdD pilus structure. The cryo-EM reconstruction is shown as  
652 surface at  $\sim 3.2\sigma$  contour level. **(E-F)** Piliation assay with single charge inversion PpdD  
653 variants. Cells (top) and sheared pilus fractions (Pili, bottom) corresponding to 0.05 OD<sub>600nm</sub>

654 of bacteria were separated on SDS-PAGE and analyzed by immunoblot using anti-PpdD  
655 antibody. PpdD residue substitutions are indicated above each lane. Migration of PpdD is  
656 indicated on the right. See also Figure S4 and Table S1.

657  
658 **Figure 4. Mapping of residues essential for pilus assembly and hypervariable regions**  
659 **on the PpdD pilus surface.**

660 **(A)** Surface electrostatic potential of the PpdD pilus **(B)** Surface representation of the PpdD  
661 pilus structure showing the distribution of charged residues essential for pilus assembly  
662 (positively charged in blue and negatively charged in red). Non-essential charged residues  
663 are colored in green. Surface of a single PpdD subunit is outlined in black. **(C)** Surface  
664 representation of a PpdD pilin subunit in the pilus colored as in (A). The surface-exposed  
665 side of the subunit (front view) displays predominantly residues permissive for charge  
666 inversions. The back view (180° rotation) shows the clustering of charged residues essential  
667 for pilus assembly on the buried face of the pilin. **(D)** Piliation assay with single charge  
668 inversion PpdD variants. Cells (top) and sheared pilus fractions (Pili, bottom) corresponding  
669 to 0.05 OD<sub>600nm</sub> of bacteria were separated on SDS-PAGE and analyzed by immunoblot  
670 using anti-PpdD antibodies. **(E)** Quantification of the fraction of PpdD assembled into pili.  
671 The bars represent the mean values and error bars represent standard deviation from 4  
672 independent experiments. Statistical analysis was performed by using ANOVA and Kruskal-  
673 Wallis multiple comparison test to compare datasets from native PpdD and its variants: ns:  
674 non-significant difference; \*\*: p<0.01; \*\*\*\*: p<0.0001. **(F)** Sphere representation of the PpdD  
675 pilus structure where the location of hypervariable regions is highlighted ( $\alpha/\beta$  loop in green,  
676  $\beta 3/\beta 4$  loop in cyan and D-region in magenta). A single PpdD subunit is outlined in black. **(G)**  
677 Schematic representation of the topological arrangement of neighboring subunits in the  
678 PpdD pilus structure. The location of hypervariable regions is indicated and colored as in (F).  
679 The size of the buried surface area in each inter-protomer interface is proportional to the  
680 width of the grey line.

681  
682 **Figure 5. Effect of charged residue substitutions on pilus assembly and on**  
683 **interactions with assembly factors in T2SS and T4PS. (A)** Pilus assembly of PpdD<sup>WT</sup> and

684 indicated variants in the T2SS (top) and T4PS (bottom panel). Cells and sheared fractions  
685 (Pili) corresponding to 0.05 OD<sub>600nm</sub> of bacteria were separated on SDS-PAGE and analyzed  
686 by immunoblot using anti-PpdD antibodies. **(B)** Bacterial two-hybrid (BACTH) analysis of  
687 interactions between PpdD<sup>WT</sup> and PpdD mutant derivatives containing charge inversion at  
688 indicated positions. Beta-galactosidase activity of *E. coli* strains DHT1 (*cyaA*) co-transformed  
689 with plasmids pCHAP8501 encoding the T18-PpdD hybrid protein and their derivatives with

690 charge inversion mutations at indicated positions and pCHAP8504 encoding T25-PpdD<sup>WT</sup>  
691 hybrid protein. (C) Interactions of PpdD and its variants with the assembly component HofN  
692 of the T4PS and PulM of the T2SS in the BACTH assay. Beta-galactosidase activity of *E. coli*  
693 DHT1 strain co-transformed with indicated plasmids encoding the T18- and T25- hybrid  
694 proteins and their mutant derivatives (listed in Table S3). BACTH analysis was performed as  
695 described previously (Nivaskumar et al., 2016). Bars represent mean values and error bars  
696 standard deviations from at least 5 independent clones. Comparison of mean values  
697 indicated for selected pairs was performed using ANOVA and Kruskal-Wallis multiple  
698 comparison test: \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001.

**Figure 6.** Normal mode analysis of T4aP filament structures. Left: Average of the atomic  
mean square fluctuations per subunit (pilin head) along the pilus axis for the first 3 non-trivial  
slowest modes (A, mode 1; B, mode 2; C, mode 3) in the EHEC (orange), Nm (red), Ng  
(blue) and Pa PAK (green) T4aP structures. For each mode, the overall direction is  
represented by the black arrows surrounding the pilus structure. (D) Representations of the  
individual modes per atom (orange arrows) on the EHEC T4aP structure (top: top view,  
bottom: side view). See also Figure S5.

**Figure 7: Comparison of T4a pili and T2SS pseudo-pilus structures.**

From left to right, pilus structure of EHEC (present work), *N. gonorrhoeae* (Wang et al.,  
*P. aeruginosa* (Wang et al., 2017), *N. meningitidis* (Kolappan et al., 2016) and *K.*  
*oxytoca* (Lopez-Castilla et al., 2017). Top row: Pilus organization with major pilin colored  
along the 1-start helix (subunits P in orange, P<sub>+1</sub> in cyan, P<sub>+2</sub> in green, P<sub>+3</sub> in red and P<sub>+4</sub> in  
magenta). Bottom row: Pilin structures in the pili. The positions of conserved Glycine  
residues (G11 for EHEC, G14 for others) and Proline 22 (P22) are labeled. See also Figures  
S7 and S8.

719 **Table 1: PpdDp NMR structure statistics and restraints** See also Figure S1.

720	<b>Number of restraints</b>	
721	NOE Distance restraints	
722	Intra-residue ( $ i-j  = 0$ )	570
723	Sequential ( $ i-j  = 1$ )	293
724	Medium-range ( $2 \leq  i-j  < 5$ )	363
725	Long-range ( $ i-j  \geq 5$ )	294
726	Ambiguous	499
727	<i>Total</i>	2219
728	Dihedral angle restraints ( $\varphi/\psi$ )	216 (108/108)
729	Hydrogen bonds restraints	30
730	RDC restraints ( $D^{NH}$ )	45
731	<b>Restraints statistics<sup>a</sup></b>	
732	RMS of distance violations	
733	NOE restraints	$0.15 \pm 0.05 \text{ \AA}$
734	H-bonds restraints	$0.22 \pm 0.04 \text{ \AA}$
735	RMS of dihedral violations	$1.47 \pm 0.11^\circ$
736	RMS of RDC violations	$1.67 \pm 0.16 \text{ Hz}$
737	Q-factor RDC	$0.098 \pm 0.003$
738	<b>RMS from idealized covalent geometry</b>	
739	bonds	$0.003 \pm 0.001 \text{ \AA}$
740	angles	$0.484 \pm 0.007^\circ$
741	impropers	$1.509 \pm 0.055^\circ$
742	<b>Structural quality<sup>a</sup></b>	
743	<i>Ramachandran statistics<sup>b</sup></i>	
744	Most favoured regions	$86.1 \pm 1.7 \%$
745	Allowed regions	$12.9 \pm 1.6 \%$
746	Generously allowed regions	$0.7 \pm 0.6 \%$
747	Disallowed regions	$0.3 \pm 0.4 \%$
748	<i>WHAT-IF Z-score<sup>c</sup></i>	
749	Backbone conformation	$-0.17 \pm 0.40$
750	2 <sup>nd</sup> generation packing quality	$-1.78 \pm 0.29$
751	Ramachandran plot appearance	$-2.46 \pm 0.28$
752	$\chi_1/\chi_2$ rotamer normality	$-2.99 \pm 0.54$
753	<b>Coordinates precision<sup>d</sup></b>	
754	All backbone atoms (26-138)	$1.51 \pm 0.35 \text{ \AA}$
755	All heavy atoms (26-138)	$1.93 \pm 0.25 \text{ \AA}$
756	All backbone atoms (secondary structures)	$0.55 \pm 0.27 \text{ \AA}$
757	All heavy atoms (secondary structures)	$1.06 \pm 0.13 \text{ \AA}$

721 <sup>a</sup> Average values and standard deviations over the 20 conformers.722 <sup>b</sup> Percentage of residues in the Ramachandran plot regions determined by PROCHECK (Laskowski et

723 al., 1993).

724 <sup>c</sup> Z-scores values reported by WHAT-IF (Vriend, 1990).725 <sup>d</sup> Average root mean square deviation (RMSD) over the 20 conformers' atomic coordinates with

726 respect to the average structure.

730 **STAR Methods**

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3 732 *Bacterial strains and plasmids*

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6 733 *Escherichia coli* strain DH5 $\alpha$  (F' *lacI*<sup>Q</sup>  $\Delta$ *lacZM15 pro+*) was used for DNA cloning purposes  
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8 734 and strains PAP7460 ( $\Delta$ (*lac-argF*)*U169 araD139 relA1 rpsL150  $\Delta$ malE444 malG501* [F' (*lacI*<sup>Q</sup>  
9  
10 735  $\Delta$ *lacZM15 pro+ Tn10*)] and BW25113 [ $\Delta$ (*araD-araB*)*567,  $\Delta$ lacZ4787(::rrnB-3),  $\lambda^-$ , rph-1,*  
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12 736  $\Delta$ (*rhaDrhaB*)*568, hsdR514*)] F' *lacI*<sup>Q</sup> (Datsenko and Wanner, 2000) were used for functional  
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14 737 assays. Strain PAP5171 (PAP7460 *degP::Km*<sup>R</sup>) was used as a host for pilus purification.  
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16 738 Bacteria were cultured in LB or M9 0.5% glycerol plates supplemented with antibiotics as  
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18 739 required: Ap (100  $\mu$ g.mL<sup>-1</sup>), Cm (25  $\mu$ g.mL<sup>-1</sup>) or Km (25  $\mu$ g.mL<sup>-1</sup>). Maltose (0.4 %) or  
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20 740 isopropyl-thio- $\beta$ -D galactoside (IPTG) was added to induce *pul* gene expression.  
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26 742 *Purification of PpdDp*

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28 743 pCHAP6154 was transformed into *E. coli* BL21(DE3) star cells (Novagen). The transformants  
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30 744 were grown at 37°C in minimal medium (M9) containing 0.1 % <sup>15</sup>N-ammonium chloride and  
31  
32 745 0.4 % <sup>13</sup>C-glucose. Protein expression was induced with 1 mM IPTG (Isopropyl  $\beta$  -D -1-  
33  
34 746 thiogalactopyranoside) when the culture reached an absorbance of 0.7–0.9 at 600 nm. After  
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36 747 3 h of induction at 30°C, the cells were centrifuged and the cell pellet was resuspended in 50  
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38 748 mM Tris–HCl buffer, pH 8.0 and disrupted by sonication. The  
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40 749 clarified cell lysate was applied to HisTrap FF column (GE Healthcare Life Sciences)  
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42 750 equilibrated with the same buffer containing 10 mM imidazole and 100 mM NaCl. Bound  
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44 751 proteins were eluted with a linear 10–300 mM imidazole gradient. The fractions containing  
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46 752 PpdD were then applied to Sephacryl S-100 column (GE Healthcare Life Sciences)  
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48 753 equilibrated with 50 mM phosphate buffer, pH 7.0, 50 mM NaCl. After the purification, the  
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50 754 fractions containing PpdD were concentrated up to 0.9 mM and supplied with 12 % D<sub>2</sub>O for  
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52 755 NMR experiments. The total protein yield was 14 mg L<sup>-1</sup>. The protein concentration was  
53  
54 756 estimated from its absorbance at 280 nm assuming a calculated  $\epsilon_{280}$  of 15,720 M<sup>-1</sup> cm<sup>-1</sup>. All  
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1 757 purification steps were performed at 4°C in the presence of the protease inhibitor cocktail  
2 758 (Roche).

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6 760 *Purification of pili*

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8 761 Bacteria of strain PAP5171 containing plasmid pCHAP8565 encoding PpdD and one of the  
9 762 two plasmids encoding the pilus assembly machinery, pCHAP8184 (for the T2SS) or pMS41  
10 763 (for T4PS), were densely inoculated on LB Cm Ap IPTG plates and cultured at 30°C for 5  
11 764 days. Bacteria were harvested in LB and pili were sheared by extensive vortexing and ten  
12 765 passages through a 26-Gauge needle. Bacteria were pelleted for 10 min at 14000 g and the  
13 766 supernatant was further pelleted in the same conditions in 1.5-ml eppendorf tubes. The  
14 767 collected cleared supernatant was subjected to ultracentrifugation for 1 hr in Ti60 Beckman  
15 768 rotor at 100 000 g. Pellets were resuspended in 50 mM HEPES pH 7.2, 50 mM NaCl and  
16 769 stored on ice.

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22 771 *Plasmid constructions*

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24 772 Plasmid pCHAP8656 contains *ppdD* gene, amplified from the genomic DNA of strain  
25 773 EDL933 using the high-fidelity Pwo DNA polymerase and placed under the control of *lacZ*  
26 774 promoter (Table S3). This plasmid, as well as the plasmid pCHAP8501 encoding the hybrid  
27 775 protein between the CyaA T18 fragment and PpdD, were used as templates for site-directed  
28 776 mutagenesis to create charged residue substitutions in PpdD listed in Table S3. For  
29 777 mutagenesis, five cycles of PCR amplification were performed in parallel with reverse and  
30 778 forward complementary oligonucleotides (custom-synthesized by Eurofins, listed in Table  
31 779 S4). The reaction mixtures were then combined and amplifications were continued for  
32 780 another 13 cycles (30 s at 96°C, 30 s at 50°C and 3 min at 72°C). After *DpnI* digestion, 10-20  
33 781 µL of the reactions were introduced into ultra-competent DH5α F'*lacI*<sup>R</sup> ultracompetent cells  
34 782 and transformants were selected on LB Cm plates. The resulting plasmids were purified  
35 783 using the Qiagen miniprep kit and verified by DNA sequencing (GATC and Eurofins).

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785 *Piliation assays*

1  
2 786 The piliation assay was performed as described previously (Luna Rico et al., 2018). Bacteria  
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4 787 of strain PAP7460 transformed with plasmid pCHAP8184 and either vector alone or  
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6 788 pCHAP8565 derivatives carrying *ppdD* were grown 48 hours at 30°C on LB agar containing  
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8 789 Ap, Cm and 0.4% maltose. Bacteria were harvested and normalized to OD<sub>600nm</sub> of 1 in LB.  
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11 790 Pili were detached by a 1-min vortex treatment and bacteria were spun for 5 min at 16000g.  
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13 791 The bacterial pellet was resuspended in SDS sample buffer at 10 OD<sub>600nm</sub>. mL<sup>-1</sup> and the  
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15 792 supernatant was cleared from the remaining bacteria in a second 10-min centrifugation. The  
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17 793 cleared supernatant was transferred to a new eppendorf tube and precipitated with 10% tri-  
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19 794 chloro-acetic acid for 30 min on ice. Pellets were collected by 30-min centrifugation at 16000  
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21 g, washed twice with acetone, air-dried and taken up in SDS sample buffer at a  
22 795  
23 concentration of 10 OD<sub>600nm</sub> equivalents per mL. Equivalent volumes of bacteria and pili  
24 796  
25 fractions were analyzed by denaturing sodium dodecyl sulfate polyacrylamide gel  
26 797  
27 electrophoresis (SDS-PAGE) with a Tris-Tricin buffer system (Schagger and von Jagow,  
28 798  
29 1987). Proteins were transferred to a nitrocellulose membrane (ECL Amersham) and probed  
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31 799  
32 with antisera generated against MalE-PpdD fusion protein described previously (Sauvonnet  
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34 et al., 2000).  
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40 803 *NMR experiments*

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42 804 NMR data were acquired at 298 K on a Varian spectrometer operating at a proton frequency  
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44 805 of 600 MHz and equipped with a cryogenically cooled triple resonance <sup>1</sup>H{<sup>13</sup>C/<sup>15</sup>N} PFG  
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46 806 probe. Proton chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate  
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48 807 (DSS) as 0 ppm. <sup>15</sup>N and <sup>13</sup>C chemical shifts were referenced indirectly to DSS (Wishart et  
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50 al., 1995). The pulse sequences were from VnmrJ Biopack. NMR data were processed with  
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52 NMRPipe/NMRDraw (Delaglio et al., 1995) and analyzed with the CcpNmr Analysis software  
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54 package (Vranken et al., 2005).  
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57 811 Residual dipolar couplings (RDCs) were determined by measuring the difference in J<sub>NH</sub>  
58  
59 812 splitting between in-phase/antiphase (IPAP) NMR experiments under isotropic and  
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61

813 anisotropic conditions (Ottiger et al., 1998). The protein sample for RDC determination was  
1  
2 814 at 1 mM in 25 mM sodium phosphate, pH 7.5, 50 mM NaCl, 12% D<sub>2</sub>O. For protein alignment  
3  
4 815 in the anisotropic sample, 10 mg/mL of the bacteriophage Pf1 (*ASLA Biotech*) was added  
5  
6 816 and the sample was incubated 1 hour in the magnetic field prior to acquisition.  
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8  
9 817 The backbone <sup>15</sup>N dynamics experiments were performed with a 1 mM sample in 50 mM  
10  
11 818 sodium phosphate, pH 7.5, 50 mM NaCl, 12% D<sub>2</sub>O. <sup>1</sup>H-<sup>15</sup>N NOE values were determined as  
12  
13 819 the ratio between the intensities of corresponding peaks in the spectra recorded with and  
14  
15 820 without pre-saturation of <sup>1</sup>H.  
16  
17 821  
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20 822 *NMR structure determination*  
21  
22 823 The structure determination strategy consisted in several rounds of automated iterative  
23  
24 824 NOESY assignment and structure calculation with the ARIA 2.3 (Rieping et al., 2007);  
25  
26 825 (Bardiaux et al., 2008) and CNS software packages (Brunger, 2007). Backbone dihedral  
27  
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29 826 angles were predicted with TALOS+ (Shen et al., 2009), and predictions classified as “good”  
30  
31 827 or “dynamic” were converted into  $\phi$  and  $\psi$  dihedral angle restraints. In addition, 45 D<sub>NH</sub>  
32  
33 828 residual dipolar coupling (RDC) were introduced as restraints in the structure calculation.  
34  
35  
36 829 Axial and rhombic components of the alignment tensor were first estimated from an  
37  
38 830 intermediate structure and further refined with the software PALES (Zweckstetter and Bax,  
39  
40 831 2000). A log-harmonic potential was employed (Nilges et al., 2008) during the cooling phase  
41  
42 832 of the simulated annealing, for NOE derived and hydrogen bonds distances restraints. This  
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44 833 potential was combined with an automated estimation of the optimal weighting of the  
45  
46 834 distance restraints (Bernard et al., 2011; Nilges et al., 2008). For NOE restraints, the final  
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48  
49 835 average weight was 14.9 kcal/mol, and 11.9 kcal/mol for hydrogen bonds restraints. The  
50  
51 836 disulfide bonds that had been confirmed by cysteine C $\beta$  chemical shifts as reported  
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53 837 previously (de Amorim et al., 2014), were imposed in the structure calculation. In the final  
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55 838 ARIA iteration, 100 conformers were calculated and the 20 lowest energy structures were  
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58 839 refined in a shell of water molecules (Linge et al., 2003). Table 1 gives a summary of NMR-  
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60 840 derived restraints and statistics on the final ensemble of NMR structure.  
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842 *Cryo-electron microscopy*

843 Three microliters PpdD pili samples were applied to plasma-cleaned lacey carbon grids and  
844 vitrified using a Vitrobot Mark IV (FEI). The grids were imaged on a Titan Krios microscope  
845 operating at 300 kV equipped with a Falcon III camera with a sampling of 1.09 Å/px. Images  
846 were collected with a defocus range of 1.0 µm to 3.0 µm. Motioncor2 was used for motion  
847 correcting all the images, followed by the CTFFIND3 program (Mindell and Grigorieff, 2003)  
848 for the defocus and astigmatism estimation. Long filament boxes were extracted from the  
849 cryo-EM images (after correction of phases and amplitudes through multiplying by the CTF)  
850 using the e2heliboxer program in EMAN2 (Tang, 2007). The SPIDER software package  
851 (Frank, 1996) was used for most other image processing. 384-px long overlapping boxes  
852 were cut from the long filament boxes (with a shift of 4% of the box size, which is ~ 1.5 times  
853 the axial rise), yielding 99,678 segments in total. A reference-based classification was used  
854 to sort the segments in terms of the axial rise and azimuthal rotation. After sorting, 25,669  
855 segments from the dominant group, which accounted for ~25% of the whole dataset, were  
856 processed by IHRSR (Egelman, 2000) to produce the final reconstruction.

857  
858 *Model building and refinement*

859 We first generated a full-length model of PpdD by adding the alpha-helical stem (F1-Q25) to  
860 the PpdDp structure by using the available structure of the full-length gonococcal PilE (PDB  
861 1AY2) as template with the Modeller program version 9v8 (Sali and Blundell, 1993). Next, the  
862 full-length PpdD model was docked as a rigid-body in the cryo-EM density map with Situs  
863 version 2.7 (Wriggers, 2012). However, only the region A23-N140 (corresponding to PpdDp  
864 plus Y24 and Q25) could be fitted inside the cryo-EM density. After symmetrization by  
865 making use of the helical symmetry parameters of the PpdD pilus cryoEM map, a theoretical  
866 density map was generated from the atomic coordinates of the fitted PpdD<sub>23-140</sub> model. A  
867 difference map was created by subtracting the theoretical PpdD<sub>23-140</sub> pilus map from the cryo-  
868 EM density map. Next, we rigidly docked the modelled PpdD<sub>1-16</sub> segment in the difference

869 map with Situs (Wriggers, 2012). An initial real-space refinement was performed with 14  
1  
2 870 copies of the docked PpdD<sub>1-16</sub> and PpdD<sub>23-140</sub> segments with PHENIX version 1.11.1 (Adams  
3  
4 871 et al., 2010). A full-length model of PpdD was then constructed with Modeller (Sali and  
5  
6 872 Blundell, 1993) by connecting the refined PpdD<sub>1-16</sub> and PpdD<sub>23-140</sub> segments by an extended  
7  
8 873 linker (S17-P22). Finally, full-atom real-space refinement, including morphing, minimization  
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10 874 and simulated-annealing, was performed with PHENIX (Adams et al., 2010) to improve  
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12 875 model geometry and the correlation with the experimental cryo-EM map. The final model of  
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14 876 PpdD pilus was validated with Molprobitry version 4.1 (Davis et al., 2007). Refinement  
15  
16 877 statistics are given in Table S5. Structure figures were generated with PyMOL (Schrödinger,  
17  
18 878 2015) and UCSF Chimera (Pettersen et al., 2004).

#### 22 879 *Analysis of residue co-evolution and secondary structure propensities*

23 880  
24 881  
25 882 Long-range contact predictions from residue co-evolution were obtained with the Gremlin tool  
26  
27 883 (Kamisetty et al., 2013). Using the EHEC PpdD sequence as query, we generated a Multiple  
28  
29 884 Sequence Alignment of 1473 homologous sequences with HHblits (Remmert et al., 2011),  
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31 885 for a final ratio of ~11 sequences per query position. Secondary structure propensities were  
32  
33 886 derived from local residue co-evolutions predicted with CCMpred (Seemayer et al., 2014),  
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35 887 following the strategy proposed recently by Toth-Petroczy *et al.* (Toth-Petroczy et al., 2016).  
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37 888 Briefly, for each position in the query sequence, a propensity score for  $\alpha$  helix or  $\beta$  strand ( $S_\alpha$   
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39 889 or  $S_\beta$ ) is calculated based on the average prediction score of short-range contacts ( $S_{i,i+1}$  to  
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41 890  $S_{i,i+4}$  for helices and  $S_{i,i+1}$  to  $S_{i,i+2}$  for strands) involving the given position or its flanking  
42  
43 891 residues (2 for helices and 1 for strands on both sides). Then, scores were normalized with  
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45 892 regards to their correlation with  $S_{i,i+1}$  scores. The final scores were obtained by subtracting  
46  
47 893 scores of non-specific contacts from scores specific to a particular secondary structure  
48  
49 894 element, *i.e.*  $S_\alpha \simeq S_{i+4} + S_{i+3} - S_{i+2} - S_{i+1}$  and  $S_\beta \simeq S_{i+2} - S_{i+1}$ .

54 895

#### 56 896 *Normal Mode Analysis of T4P structures*

897 Normal modes were computed with Prody (Bakan et al., 2014) with an Anisotropic Network  
1  
2 898 Model on C $\alpha$  atoms of T4P filament structures and a cutoff of 15 Å for pairwise interactions in  
3  
4 899 the elastic network. The different helical pitch values of the EHEC, Nm, Ng and PAK T4P  
5  
6 900 filaments lead to structures with significantly variable length for a fixed number of pilin  
7  
8 901 subunits, that may influence the outcome of the normal mode analysis. Consequently,  
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10 902 filament structures of equivalent length were generated using different number of subunits  
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12 903 (30 for Nm and Ng, 29 for PAK and 27 for EHEC), the respective helical symmetry and the  
13  
14 904 pilin structure. The following PDB entries were used: 5KUA for Nm, 5VXX for Ng and 5VXY  
15  
16 905 for PAK pili. For each of the first 3 non-trivial lowest frequency modes, the average of the  
17  
18 906 atomic mean square displacements was calculated for each subunit, excluding the first 24  
19  
20 907 residues.  
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24 908  
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26 909

## 28 910 **Supplemental tables and figures**

29 911  
30 912 **Table S1; Related to Figure 3:** Agreement of high confidence (Prob > 0.7) predicted  
31  
32 913 contacts from residue co-evolution with PpdD pilus model.  
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34 914  
35 915 **Table S2; Related to Figure 1:** Sequence and structure similarity between EHEC PpdD and  
36  
37 916 other T4a pilins  
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39 917  
40 918 **Table S3; Related to STAR Methods:** Plasmids used in this study.  
41

42 919  
43 920 **Table S4; Related to STAR Methods:** Oligonucleotides used in this study.  
44

45 921  
46 922 **Table S5; Related to STAR Methods:** Refinement statistics of the PpdD pilus structure  
47

48 923  
49 924 **Figure S1; Related to Table 1:** NOE restraints of the PpdDp NMR structure.  
50

51 925  
52 926 **Figure S2; Related to Figure 1:** (A) <sup>1</sup>H-<sup>15</sup>N heteronuclear NOE of PpdDp in solution.  
53

54 927  
55 928 **Figure S3; Related to Figure 2:** (A,B) Large variability in terms of the helical symmetry of  
56  
57 929 PpdD pili.  
58

59 930  
60 931 **Figure S4; Related to Figure 3:** EHEC PpdD pilus contact map and evolutionary contact  
61  
62 932 predictions.  
63

64 933  
65 934 **Figure S5; Related to Figure 6:** Buried surface areas of inter-subunit interfaces in T4Pa  
66  
67 935 structures.  
68  
69 936

937 **Figure S6; Related to Figure 1:** Structural comparison of major T4a pilins (periplasmic  
1 938 domains).

2 939

3 940 **Figure S7; Related to Figure 7:** Secondary structure elements in T4a pilins.

4 941

5 942 **Figure S8; Related to Figure 7:** Surface electrostatics potential of T4P pilus structure.

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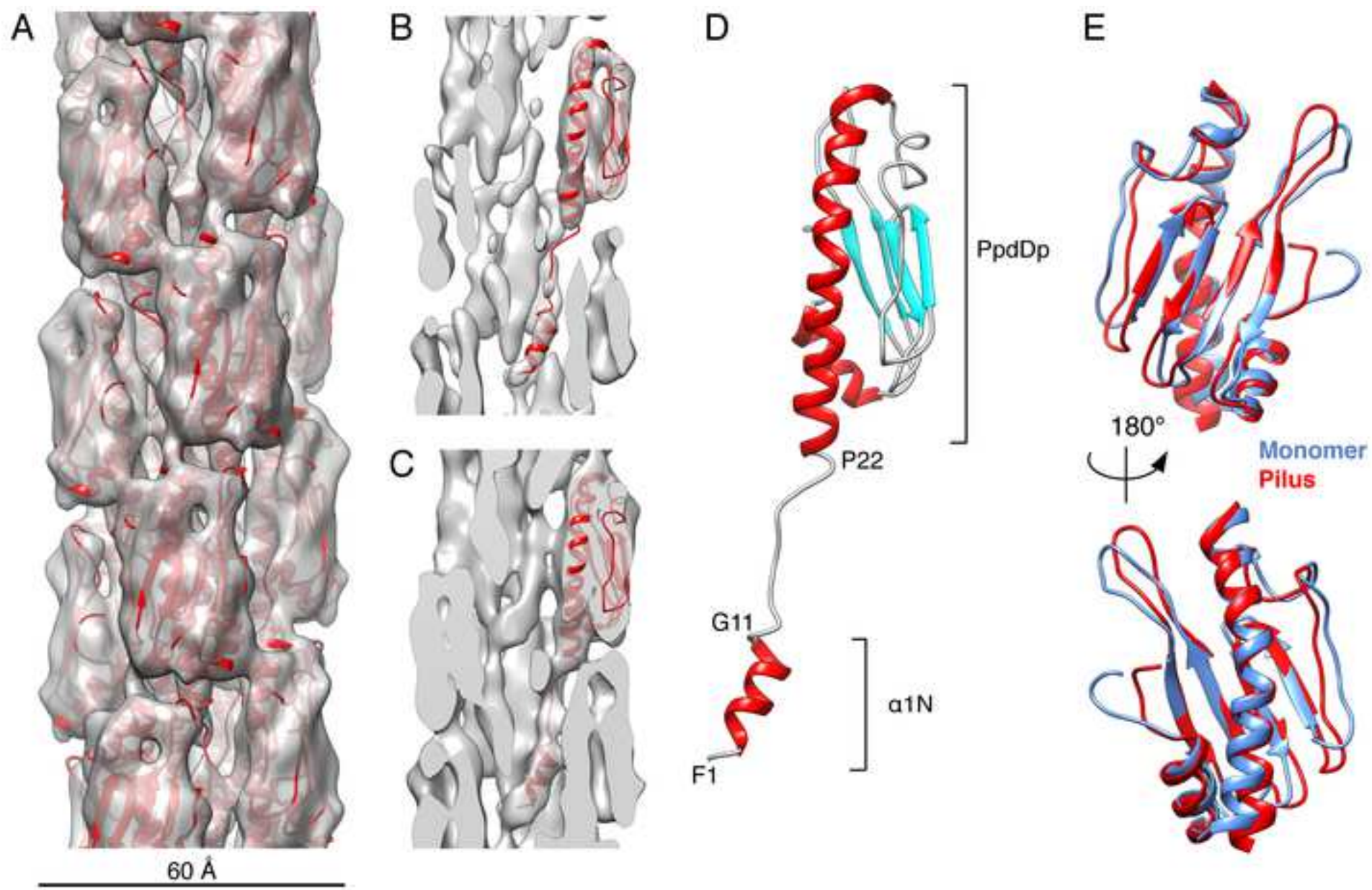
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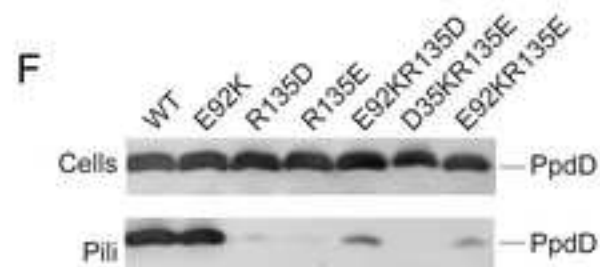
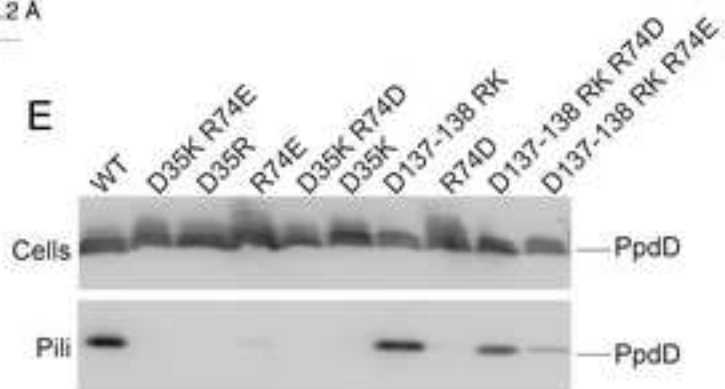
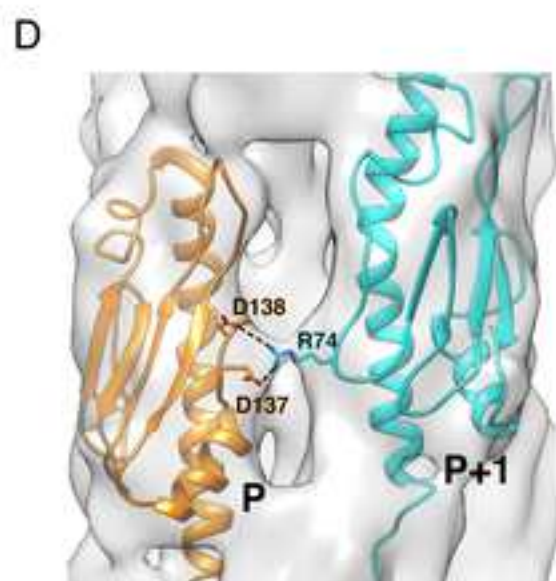
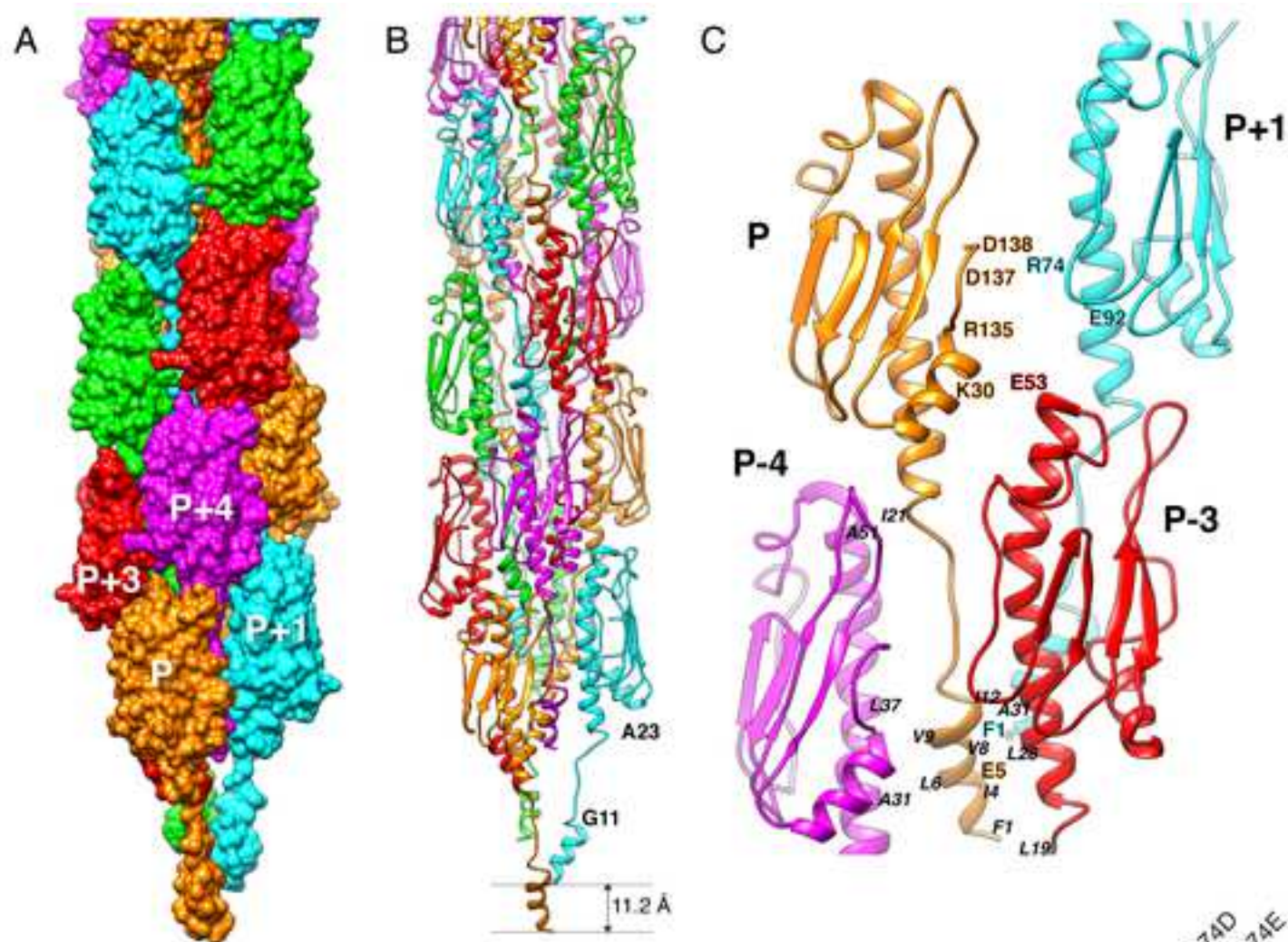
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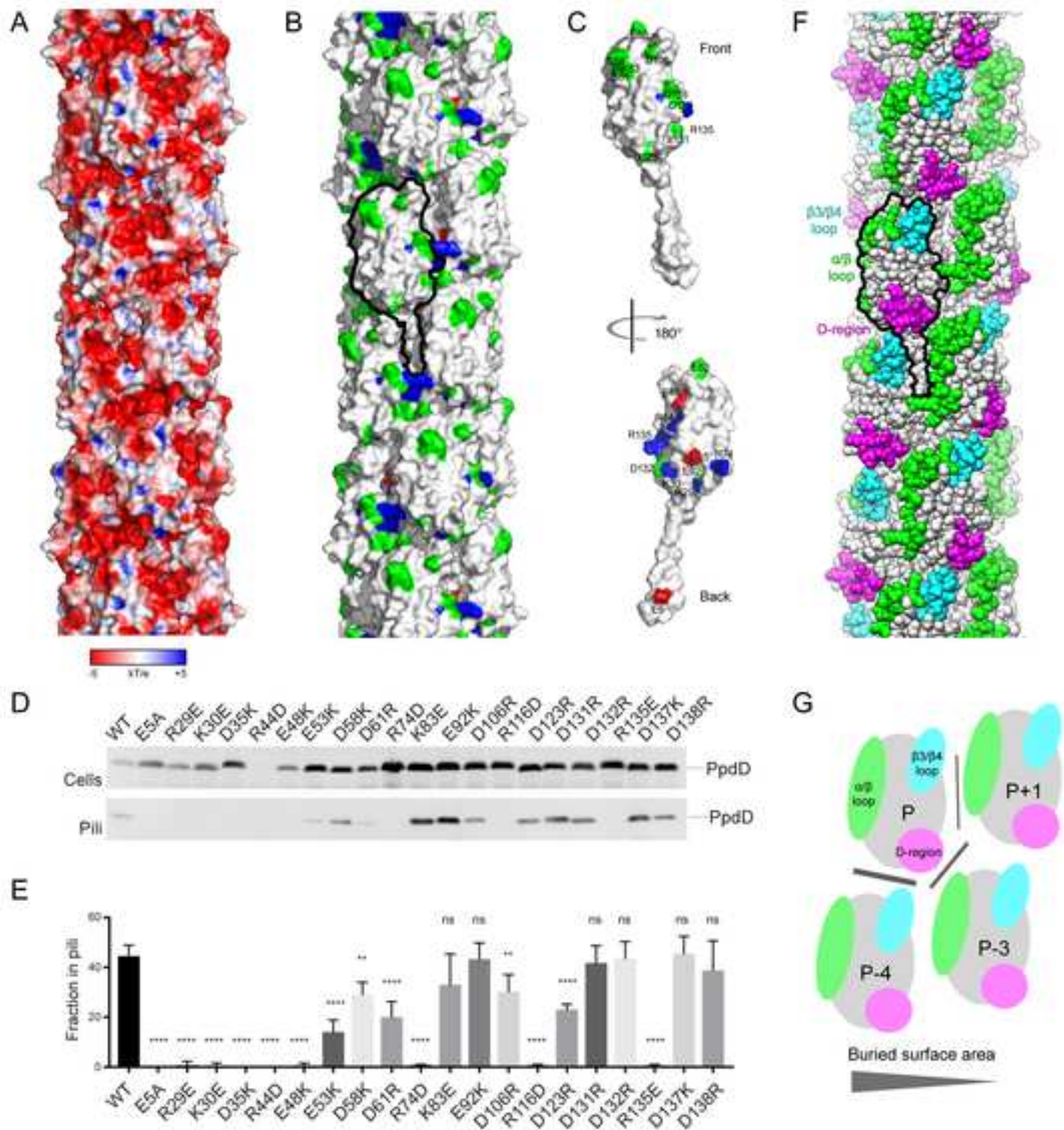
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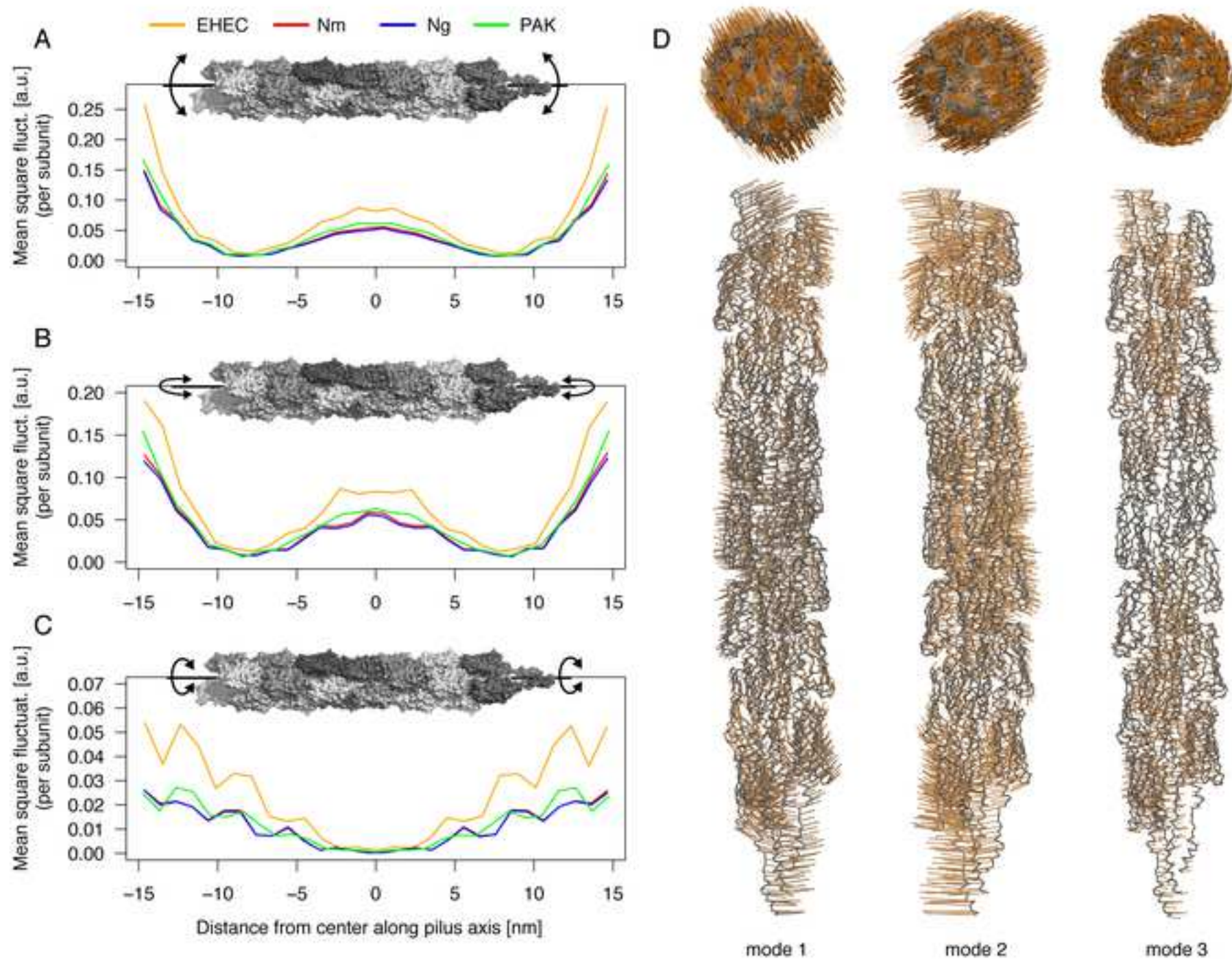


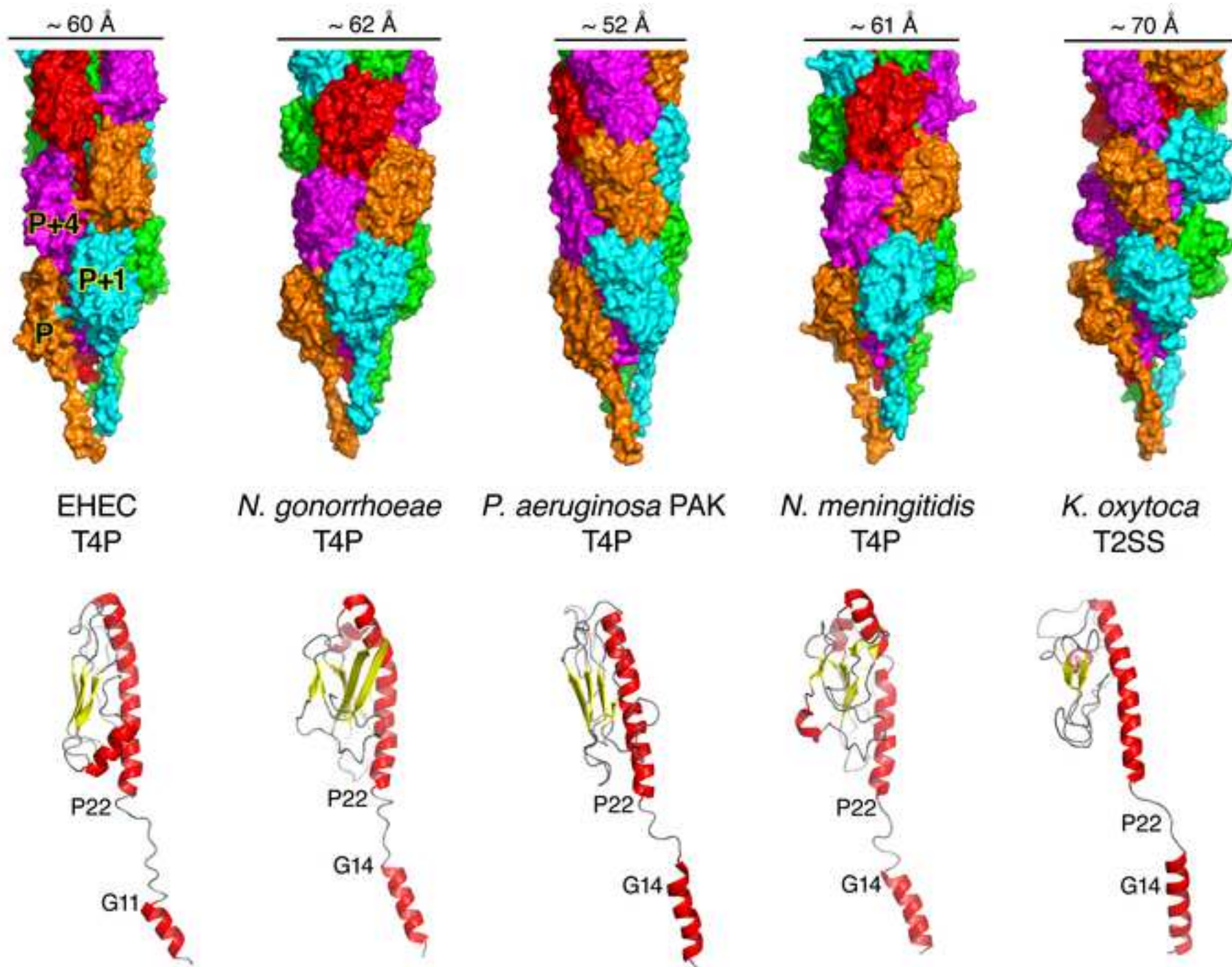












**TABLE FOR AUTHOR TO COMPLETE**

Please upload the completed table as a separate document. **Please do not add subheadings to the Key Resources Table.** If you wish to make an entry that does not fall into one of the subheadings below, please contact your handling editor. (NOTE: For authors publishing in *Current Biology*, please note that references within the KRT should be in numbered style, rather than Harvard.)

**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-MalE-PpdD rabbit polyclonal antibodies	(Sauvonnet et al. 2000)	N/A
ECL™ anti-rabbit IgG linked to horse radish peroxidase from donkey	Amersham	Cat# NA934V
<b>Bacterial Strains</b>		
<i>Escherichia coli</i> DH5 $\alpha$ F' <i>lacI</i> <sup>Q</sup>	Laboratory collection	N/A
<i>Escherichia coli</i> BW25113 F' <i>lacI</i> <sup>Q</sup>	(Datsenko and Wanner, 2000)	N/A
<i>Escherichia coli</i> BL21(DE3) Star	Novagen	Cat# 69450
<i>Escherichia coli</i> PAP7460	(Possot et al., 2000)	N/A
<i>Escherichia coli</i> DHT1	(Dautin et al., 2000)	N/A
<i>Escherichia coli</i> PAP5171	(Francetic and Pugsley, 2005)	N/A
<b>Biological Samples</b>		
Sheared pilus fraction from strain PAP5171 carrying plasmids pCHAP8565 and pCHAP8184	This study	N/A
Sheared pilus fraction from strain PAP5171 carrying plasmids pCHAP8565 and pMS41	This study	N/A
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
DNA polymerase Pwo	Roche	Cat#11644947001
T4 DNA ligase	New England Biolabs	Cat# M0202
PpdD(25-140)Thr-His <sub>6</sub> purified protein	(de Amorim et al, 2014)	N/A
Q5 High Fidelity Polymerase	New England Biolabs	Cat# M0491
<i>KpnI</i> -HF restriction enzyme	New England Biolabs	Cat# R3142L
<i>EcoRI</i> -HF restriction enzyme	New England Biolabs	Cat# R3010L
<i>Complete protease inhibitor</i>	Roche	Cat#11836153001
Bacteriophage Pf1	ASLA biotech	Cat# P-50-P
D <sub>2</sub> O	Eurisotop	Cat# D214F
<b>Critical Commercial Assays</b>		
Qiaprep Spin plasmid purification kit	Qiagen	Cat#: 27104
Qiaquick PCR purification kit	Qiagen	Cat#: 28104
Pierce®ECL 2 Western Blotting Substrate	Fisher Scientific	Cat# : 993PT
<b>Deposited Data</b>		
NMR resonance assignments	(de Amorim et al., 2014)	BMRB: 18823
NMR structure ensemble of truncated PpdD pilin of EHEC	This paper	PDB: 6GMS
Cryo-EM reconstruction of the EHEC type IV pilus	This paper	EMDB-0070
Atomic model of the EHEC type IV pilus	This paper	PDB: 6GV9

Oligonucleotides		
See <a href="#">Table S4</a> for list of primers used in this study	N/A	NA/
Recombinant DNA		
See <a href="#">Table S3</a> for list of plasmids used in this study	N/A	N/A
Software and Algorithms		
PRISM		
Image J		
VnmrJ Biopack	Agilent Technologies, Inc.	N/A
NMRPipe/NMRDraw	(Delaglio et al., 1995)	<a href="https://spin.niddk.nih.gov/NMRPipe/">https://spin.niddk.nih.gov/NMRPipe/</a>
CcpNmr Analysis 2.4	(Vranken et al., 2005)	<a href="https://www.ccpn.ac.uk/">https://www.ccpn.ac.uk/</a>
ARIA 2.3	(Rieping et al., 2007)	<a href="http://aria.pasteur.fr">http://aria.pasteur.fr</a>
CNS 1.2	(Brunger, 2007)	<a href="http://cns-online.org">http://cns-online.org</a>
TALOS+	(Shen et al., 2009)	<a href="https://spin.niddk.nih.gov/NMRPipe/talos/">https://spin.niddk.nih.gov/NMRPipe/talos/</a>
PALES	(Zweckstetter and Bax, 2000)	<a href="https://spin.niddk.nih.gov/bax/software/PALLES/">https://spin.niddk.nih.gov/bax/software/PALLES/</a>
CTFFIND3	(Mindell and Grigorieff, 2003)	<a href="http://grigoriefflab.janelia.org/ctf">http://grigoriefflab.janelia.org/ctf</a>
EMAN2	(Tang, 2007)	<a href="https://blake.bcm.edu/emanwiki/EMAN2">https://blake.bcm.edu/emanwiki/EMAN2</a>
SPIDER	(Frank, 1996)	<a href="https://spider.wadsworth.org/">https://spider.wadsworth.org/</a>
IHRSR	(Egelman, 2000)	<a href="https://cryoem.ucsd.edu/wikis/software/start.php?id=ihrsr">https://cryoem.ucsd.edu/wikis/software/start.php?id=ihrsr</a>
Modeller 9v8	(Sali and Blundell, 1993)	<a href="https://salilab.org/modeller/">https://salilab.org/modeller/</a>
PHENIX 1.11	(Adams et al., 2010)	<a href="https://www.phenix-online.org/">https://www.phenix-online.org/</a>
Situs 2.7	(Wriggers, 2012)	<a href="https://situs.biomachina.org/">https://situs.biomachina.org/</a>
Molprobit 4	(Davis et al., 2007)	<a href="http://molprobity.biochem.duke.edu/">http://molprobity.biochem.duke.edu/</a>
Pymol 2	(Schrödinger, 2015)	<a href="https://pymol.org/">https://pymol.org/</a>
UCSF Chimera	(Pettersen et al., 2004)	<a href="https://www.cgl.ucsf.edu/chimera/">https://www.cgl.ucsf.edu/chimera/</a>
WHAT-IF	(Vriend, 1990)	<a href="https://swift.cmbi.umcn.nl/whatif/">https://swift.cmbi.umcn.nl/whatif/</a>
Procheck	(Laskowski et al., 1993)	<a href="https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/">https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/</a>
Gremlin	(Kamisetty et al., 2013)	<a href="http://gremlin.bakerlab.org/">http://gremlin.bakerlab.org/</a>
CCMpred	(Seemayer et al., 2014)	<a href="https://github.com/soedinglab/CCMpred">https://github.com/soedinglab/CCMpred</a>
Prody	(Bakan et al., 2014)	<a href="http://prody.csb.pitt.edu/">http://prody.csb.pitt.edu/</a>

# Structure and assembly of the Enterohemorrhagic

## *Escherichia coli* type 4 pilus

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### Supplementary Information:

Supplementary Tables 1-5.

Supplementary Figures 1-8.

Supplementary references.

**Table S1; Related to Figure 3:** Agreement of high confidence predicted contacts from residue co-evolution with PpdD pilus model. The accuracy (number of contacts with Prob > 0.7 consistent with the model/total number of contacts) is reported for increasing distance thresholds and for different rotameric states of the PpdD pilus model. A contact is considered as consistent with the model if the shortest distance between any non-hydrogen atoms of the two involved residues is smaller than the threshold. Optimized side-chain rotamers were built with SCWRL4.0 (Krivov et al., 2009). Alternatively, the at-most 10 best (most frequent) rotameric states from the Dunbrack library (Shapovalov and Dunbrack, 2011) were considered simultaneously to measure inter-residue distances in the PpdD pilus model.

<b>Distance threshold (Å)</b>	<b>PpdD pilus model (PDB 6GV9)</b>	<b>PpdD pilus model + optimized side-chain rotamers</b>	<b>PpdD pilus model + 10 best side-chain rotamers</b>
5	46.8 %	47.5 %	60.3 %
8	79.4 %	79.4 %	83.0 %
10	88.7 %	89.4 %	90.1 %
12	91.5 %	92.9 %	94.3 %

**Table S2; Related to Figure 1:** Sequence and structure similarity between EHEC PpdD and other T4a pilins

Pilin*	PDB id	% identity (full-length)	% identity (from Pro 22)	RMSD (Å)#
Ft PilE	3SOJ	25	16	2.7
Pa K122-4 PilA	1QVE	31	23	2.8
Pa PAK PilA	1OQW	30	22	2.8
Dn FimA	3SOK	28	20	3.0
Pa 110594 PilA	3JYZ	26	18	3.1
Ab PilA	5IHJ	30	20	3.1
Nm PilE	5JW8	32	26	3.2
Ng PilE	1AY2	32	26	3.2

\* Ft: *Francisella tularensis*; PaK122-4: *Pseudomonas aeruginosa* strain K122-4; PAK: *Pseudomonas aeruginosa* strain PAK; Dn: *Dichelobacter nodosus*; Pa110594: *Pseudomonas aeruginosa* strain Pa110594; Ab: *Acinetobacter baumannii*; Nm: *Neisseria meningitidis*; Ng: *Neisseria gonorrhoeae*.

# Computed with TM-align (Zhang and Skolnick, 2005).

**Table S3; Related to STAR Methods: Plasmids used in this study.**

Plasmid name	Ori/resistance <sup>1</sup>	Relevant markers	Source/reference
pCHAP8184	ColE1/Ap <sup>R</sup>	<i>pulS,pulAB pulCDEFHIJKLMNO</i>	(Campos <i>et al.</i> , 2010)
pSU18	p15A/Cm <sup>R</sup>	<i>placZ-lacZ<math>\alpha</math></i>	(Bartolome <i>et al.</i> , 1991)
pUC18	ColE1/Ap <sup>R</sup>	<i>placZ-lacZ<math>\alpha</math></i>	(Yanisch-Perron <i>et al.</i> , 1985)
pCHAP6154	ColE1/Ap <sup>R</sup>	<i>pT7-peIBsp-ppdD(25-140)Thr His<sub>6</sub></i>	(de Amorim <i>et al.</i> , 2014)
pCHAP8565	p15A/Cm <sup>R</sup>	<i>ppdD EHEC</i>	(Luna Rico <i>et al.</i> , 2019)
pCHAP6263	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E5A</sup></i>	This study
pCHAP6250	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D35R</sup></i>	This study
pCHAP6254	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R29E</sup></i>	This study
pCHAP6256	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E131K</sup></i>	This study
pCHAP6257	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E92K</sup></i>	This study
pCHAP6258	p15A/Cm <sup>R</sup>	<i>ppdD<sup>K83E</sup></i>	This study
pCHAP6260	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R74D</sup></i>	This study
pCHAP6261	p15A/Cm <sup>R</sup>	<i>ppdD<sup>K30E</sup></i>	This study
pCHAP6264	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R44D</sup></i>	This study
pCHAP6265	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D106R</sup></i>	This study
pCHAP6266	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R116D</sup></i>	This study
pCHAP6267	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D132R</sup></i>	This study
pCHAP6268	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E48K</sup></i>	This study
pCHAP6269	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E53K</sup></i>	This study
pCHAP6317	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D58K</sup></i>	This study
pCHAP6271	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D61R</sup></i>	This study
pCHAP6272	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D123R</sup></i>	This study
pCHAP6316	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D35K</sup></i>	This study
pCHAP6320	p15A/Cm <sup>R</sup>	<i>ppdD<sup>K30D</sup></i>	This study
pCHAP6333	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R74E</sup></i>	This study
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pCHAP6259	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R135E</sup></i>	This study
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pCHAP6314	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E92R</sup></i>	This study
pCHAP6257	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E92K</sup></i>	This study
pCHAP6259	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R135E</sup></i>	This study
pCHAP6322	p15A/Cm <sup>R</sup>	<i>ppdD<sup>R135D</sup></i>	This study
pCHAP6277	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E92K R135E</sup></i>	This study
pCHAP6285	p15A/Cm <sup>R</sup>	<i>ppdD<sup>E92K R135D</sup></i>	This study
pCHAP8956	p15A/Cm <sup>R</sup>	<i>ppdD<sup>D35K R135E</sup></i>	This study
pUT18c	ColE1/Ap <sup>R</sup>	<i>placUV5-cyaA</i> T18 fragment in pUC18	(Karimova <i>et al.</i> , 1998)
pKT25	p15A/Km <sup>R</sup>	<i>placUV5-cyaA</i> T25 fragment in pSU38	(Karimova <i>et al.</i> , 1998)
pCHAP8501	ColE1/Ap <sup>R</sup>	<i>T18-PpdD</i>	(Luna Rico <i>et al.</i> , 2019)
pCHAP8504	p15A/Km <sup>R</sup>	<i>T25-PpdD</i>	(Luna Rico <i>et al.</i> , 2019)

pCHAP8973	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>E5A</sup>	This study
pCHAP8768	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>R29E</sup>	This study
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pCHAP8772	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D35K</sup>	This study
pCHAP8774	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>R44D</sup>	This study
pCHAP8776	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>E48K</sup>	This study
pCHAP8916	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D58K</sup>	This study
pCHAP8780	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D61K</sup>	This study
pCHAP8782	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>R74D</sup>	This study
pCHAP8784	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>R116D</sup>	This study
pCHAP8786	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>R135E</sup>	This study
pCHAP8920	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>K83E</sup>	This study
pCHAP8922	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>E92K</sup>	This study
pCHAP8924	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D106R</sup>	This study
pCHAP8926	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D123R</sup>	This study
pCHAP8928	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>E131R</sup>	This study
pCHAP8930	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D132R</sup>	This study
pCHAP8936	ColE1/Ap <sup>R</sup>	T18-PpdD <sup>D138R</sup>	This study
pCHAP8155	p15A/Km <sup>R</sup>	T25-PulM	(Nivaskumar et al., 2016)
pCHAP8910	p15A/Km <sup>R</sup>	T25-HofN	(Luna Rico et al., 2019)
pMS41	ColE1/Ap <sup>R</sup>	EHEC T4PS ( $\Delta$ ppdD,hofBC, hofMNOPQ,ppdABYgdBppdC gspO)	(Luna Rico et al., 2019)

<sup>1</sup>Ap<sup>R</sup>, ampicillin-resistance; Cm<sup>R</sup>, chloramphenicol-resistance, Km<sup>R</sup>, kanamycin-resistance).

**Table S4; Related to STAR Methods: Oligonucleotides used in this study.**

Oligonucleotide name	Sequence (5'-3')
PpdD-SD-EcoRI	CTATTCTGAATTCAAAGTAGCGCCAACCAAATC
PpdD-Hind-3	cacAAGCTTGCATGCCTGCAGGTCGACTCTAG
HofC EcoRI 5	CACGAATTCTGAAGAGTTAATCCGCGTATTG
HofC Hind 3	GACTGCAAGCTTCGTTATCCCATCCCACTCATCG
HofM Pst 5	CGCCTGCAGGGTAGTATAAAGGCAAGC
HofQ Sph 3	CACGCATGCCACGTTTACGCGTAAAAAC
PpdD 5 Kpn	GCAGGTACCTATGACACTTATCGAACTGATGGTG
PpdD 3 Eco	CACGAATTCCATTTTCACTGAGCTGTGGAAT
PpdDE5A 5 Kpn	GCAGGTACCTATGACACTTATCGcACTGATGGTG
PpdD D35R 5	CAAAGCCGCACTCACCcgCATGCTACAAAC
PpdD D35R 3	GTTTGTAGCATGcgGGTGAGTGCGGCTTTG
PpdD E5A 5	GTTTTACACTTATCGcACTGATGGTGG
PpdD E5A 3	CCACCATCAGTgCGATAAGTGTA AAC
PpdD R29E 5	CAA AACTACCTGgaaAAAGCCGCACTC
PpdD R29E 3	GAGTGCGGCTTTtcCAGGTAGTTTTG
PpdD R29D 5	TATCAA AACTACCTGgaCAAAGCCGCACTCACC
PpdD R29D 3	GGTGAGTGCGGCTTTGtcCAGGTAGTTTTGATA
PpdD K30E 5	ACTACCTGCGCgAAGCCGCACTC
PpdD K30E 3	GAGTGCGGCTTcGCGCAGGTAGT
PpdD K30D 5	CAA AACTACCTGCGCgAtGCCGCACTCACCGAC
PpdD K30D 3	GTCGGTGAGTGCGGCaTcGCGCAGGTAGTTTTG
PpdD R44D 5	CCTTTGTGCCTTACgaTACCGCCGTAGAG
PpdD R44D 3	CTCTACGGCGGTAtcGTAAGGCACAAAGG
PpdD K30D 5	CAA AACTACCTGCGCgAtGCCGCACTCACCGAC
PpdD K30D 3	GTCGGTGAGTGCGGCaTcGCGCAGGTAGTTTTG
PpdD D35K 5	AAAGCCGCACTCACCaAaATGCTACAAACCTTTG
PpdD D35K 3	CAAAGGTTTGTAGCATtTtGGTGAGTGCGGCTTT
PpdD R74D 5	CCTACCACCACCgaCTATGTTTCAGCC
PpdD R74D 3	GGCTGAAACATAGtcGGTGGTGGTAGG
PpdD R74E 5	CCCTCGCCTACCACCACCgaaTATGTTTCAGCCATGAGTG
PpdD R74E 3	CACTCATGGCTGAAACATAttcGGTGGTGGTAGGCGAGGG
PpdD D106R 5	CATGACGCCAGGTTGGcgTAACGCAAACGGCGTCAC
PpdD D106R 3	GTGACGCCGTTTGCCTTAacgCAAACCTGGCGTCATG
PpdD D106K 5	ATGACGCCAGGTTGGaAaAACGCAAACGGCGTC
PpdD D106K 3	GACGCCGTTTGCCTTtTtCCAACCTGGCGTCAT
PpdD D137K 5	GTCTTCCGCTTTaAaGACGCCAACTAAG
PpdD D137K 3	CTTAGTTGGCGTctTtAAAGCGGAAGAC
PpdD D138R 5	GTCTTCCGCTTTGATcgCGCCAACTAAGG
PpdD D138R 3	CCTTAGTTGGCGcgATCAAAGCGGAAGAC
PpdD D137K D138R 5	AAGATGTCTTCCGCTTTaAacgGCCAACTAAGGAGC
PpdD D137K D138R 3	GCTCCTTAGTTGGCgctTtAAAGCGGAAGACATCTT
PpdD E131K 5	CAGCAAGCCTGCaAAGATGTCTTCC
PpdD E131K 3	GGAAGACATCTTtGCAGGCTTGCTG
PpdD E92K 5	CTGACCGGGCAaAgAGTCTCAATGG
PpdD E92K 3	CCATTGAGACTcTtTTGCCCGGTCAG
PpdD K83E 5	CATGAGTGTGGCAgAGGGCGTGGTG
PpdD K83E 3	CACCACGCCCTcTGCCCACTCATG

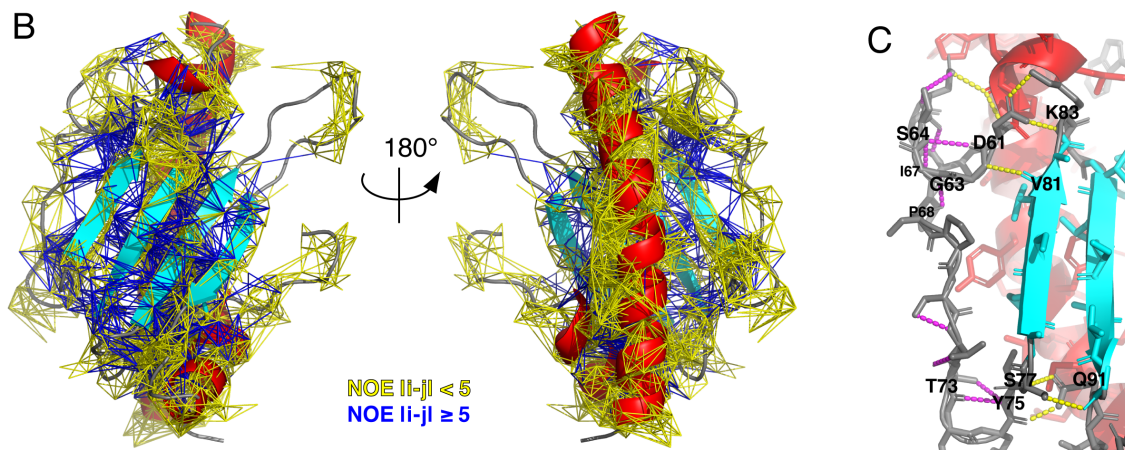
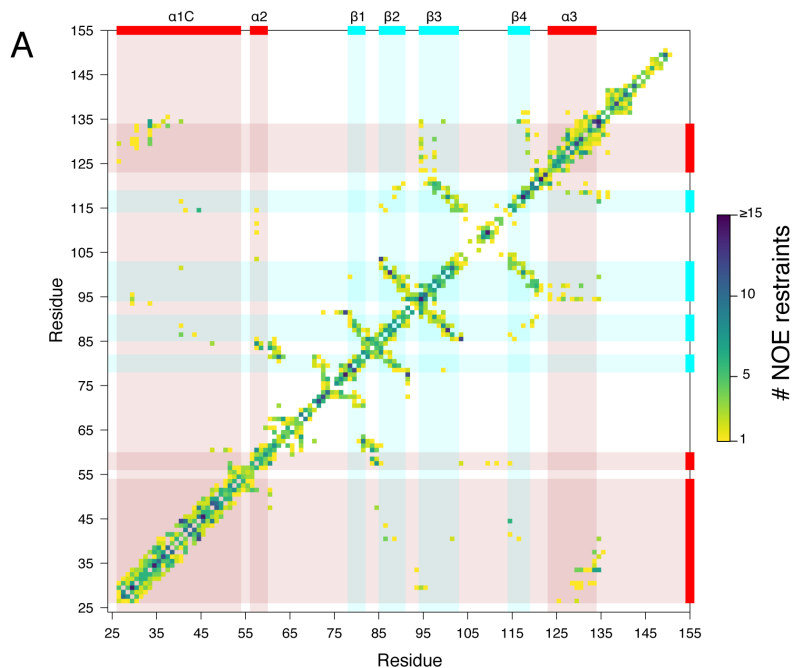
PpdD D132R 5	GCAAGCCTGCGAACGTGTCTTCCGCTTTGATGACG
PpdD D132R 3	CGTCATCAAAGCGGAAGACACGTTTCGCAGGCTTGC
PpdD D123R 5	GCAATATTCAAAGTCGCAGCGCATTGCAGC
PpdD D123R 3	GCTGCAATGCGCTGCGACTTTGAATATTGC
PpdD D61R 5	GGATTAGATACCTGCCGCGGTGGCAGCAATGG
PpdD D61R 3	CCATTGCTGCCACCGCGGCAGGTATCTAATCC
PpdD R135E 5	GAAGATGTCTTCgaATTcGATGACGCCAAC
PpdD R135E 3	GTTGGCGTCATCgAATtcGAAGACATCTTC
PpdD E48K 5	CCTTACCGTACCGCCGTAAAGTTGTGCGCGCTGG
PpdD E48K 3	CCAGCGCGCACAACTTTACGGCGGTACGGTAAGG
PpdD E53K 5	GGTATCTAATCCACCATGTTTCAGCGCGCACAACTCTACG
PpdD E53K 3	GCCACCGTCGCAGGTtTtTAATCCACCATGTTC
PpdD D58K 5	GAACATGGTGGATTAaAaACCTGCGACGGTGGC
PpdD D58K 3	CCACCGTCGCAGGTACGTAATCCACCATGTTC
PpdD R116D 5	CGTCACCGGCTGGGCGgaCAACTGCAATATTCAAAG
PpdD R116D 3	CTTTGAATATTGCAGTTGtcCGCCCAGCCGGTGACG
PpdD R44E E48K 5	CCTTACgaaACCGCCGTaAAGTTGTGCGCGCTGG
PpdD R44E E48K 3	CCAGCGCGCACAACTtTACGGCGGTttcGTAAGG
PpdD R44D E48R 5	GCCTTACgaTACCGCCGTAcgtTTGTGCGCGCTGGAACATG
PpdD R44D E48R 3	CATGTTCCAGCGCGCACAAacgTACGGCGGTAtcGTAAGGC

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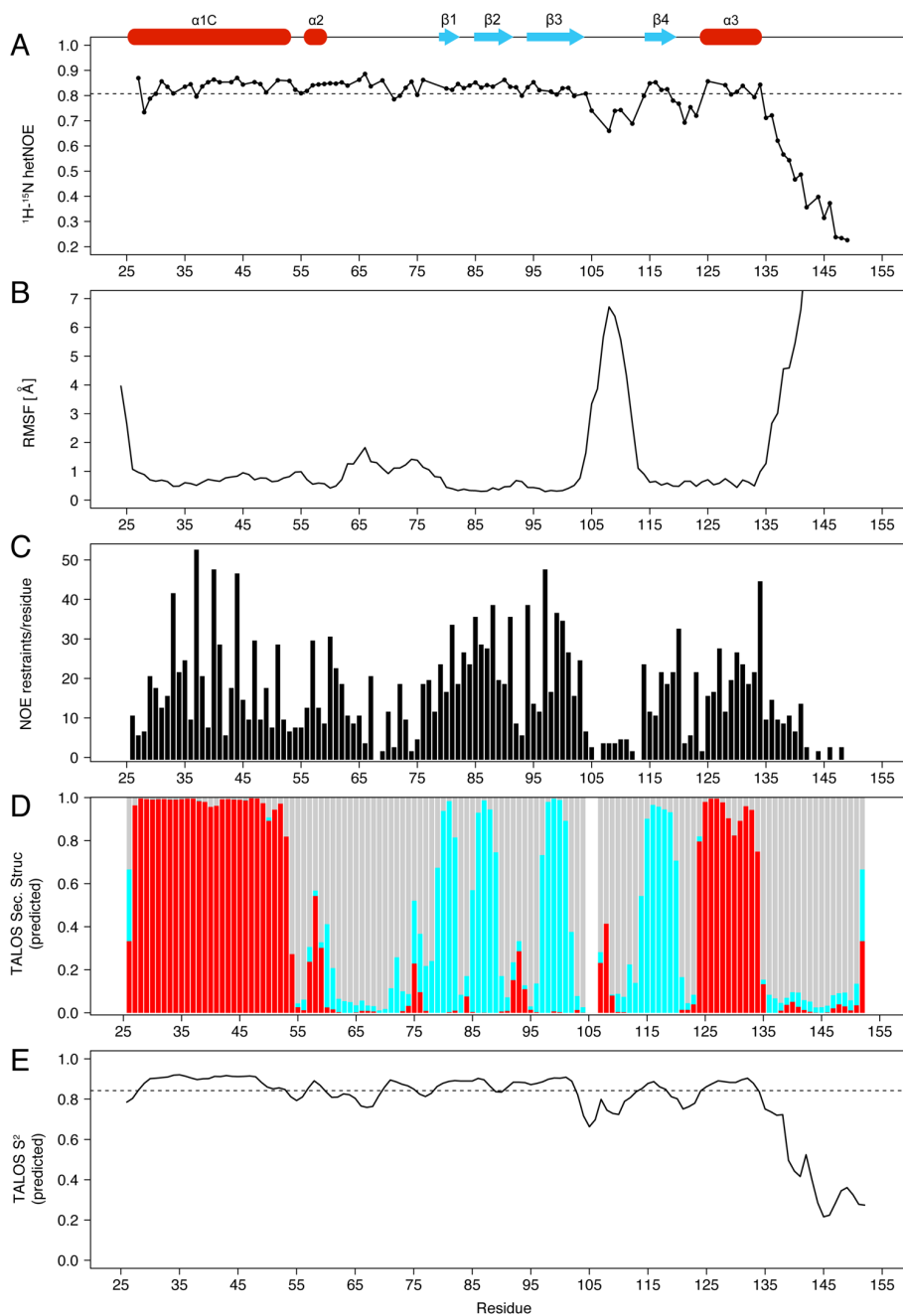
**Table S5; Related to STAR Methods:** Refinement statistics of the PpdD pilus structure

<b>Deviation from ideal geometry</b>	
RMS <sup>a</sup> for bond lengths	0.02 Å
RMS for bond angles	2.40 °
Cβ deviations	0 %
<b>Molprobit statistics</b>	
Ramachandran favored	92.0 %
Ramachandran allowed	6.5 %
Ramachandran outliers	1.4 %
Rotamer outliers	0 %
Clashscore	28.7
Overall score	2.43

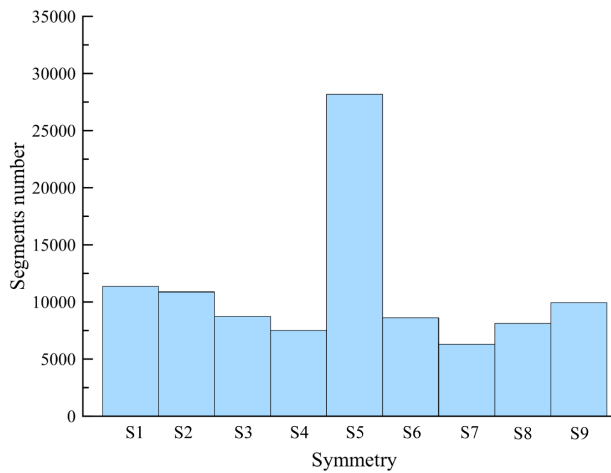
<sup>a</sup>RMS, root mean square



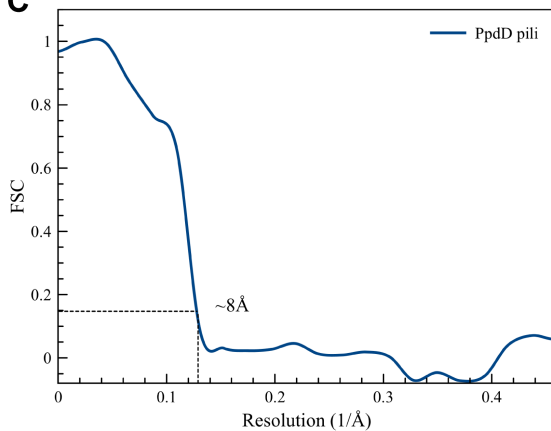
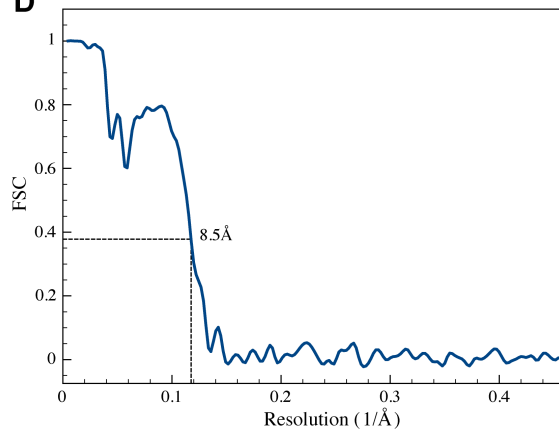
**Figure S1; Related to Table 1: NOE restraints of the PpdDp NMR structure.** (A) Map of inter-residue NOE restraints used to calculate the PpdDp structure. The color scale corresponds to the number of unique NOE restraints for a pair of residues. Secondary structures are shown in red (helix) and cyan (strands). (B) NOE restraints shown on the lowest energy conformer of the PpdDp NMR structure. Long range NOE restraints are shown with blue lines and other NOE restraints with yellow lines. (C) Hydrogen-bonds detected in the PpdDp structure stabilizing the  $\alpha/\beta$  loop.



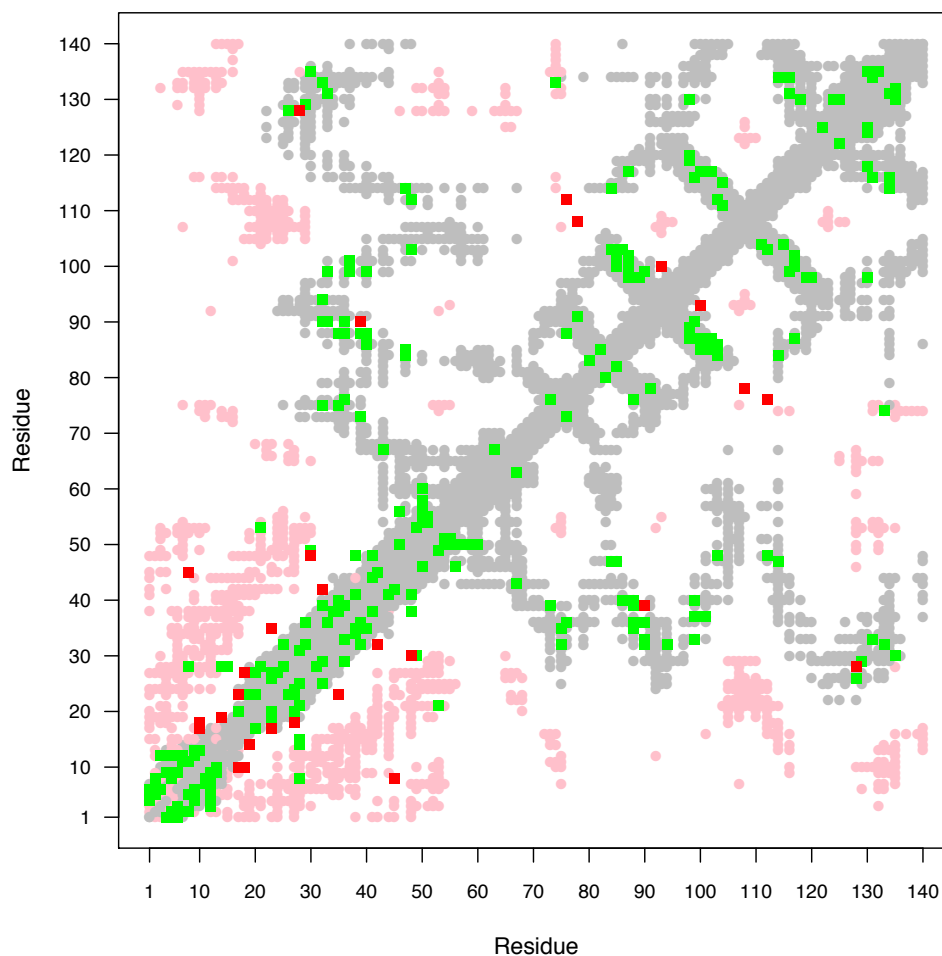
**Figure S2; Related to Figure 1: Dynamics and flexibility of PpdDp in solution** (A)  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear NOE of PpdDp in solution. Secondary structure elements are shown on top (helix in red, strand in blue). (B) Ensemble Root Mean Square Fluctuation (RMSF) of the NMR PpdDp bundle. (C) Number of NOE ( $|i-j| > 1$ ) restraints per residue as assigned by ARIA. (D) Secondary structure (probabilities) predicted from chemical shifts using TALOS+ (red: helix, cyan: strand, grey: loop). (E) TALOS+ predicted order parameter  $S^2$ .

**A****B**

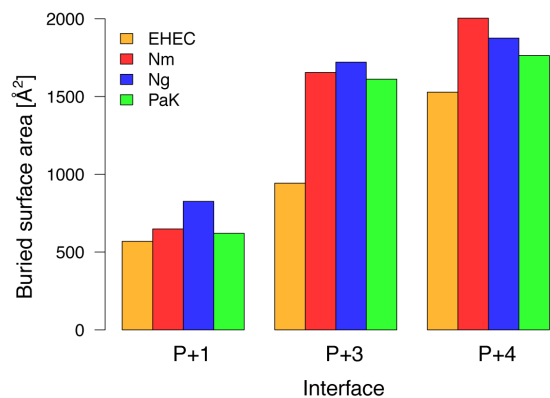
Symmetry	Twist	Rise
S1	94°	8.8Å
S2	96°	8.8Å
S3	98°	8.8Å
S4	94°	10.8Å
S5	96°	10.8Å
S6	98°	10.8Å
S7	94°	12.8Å
S8	96°	12.8Å
S9	98°	12.8Å

**C****D**

**Figure S3; Related to Figure 2: Large variability in terms of the helical symmetry of PpdD pili.** (A) Histogram showing the symmetry distribution of PpdD pili segments. Nine reconstructions were generated with symmetry parameters shown in (B). These volumes were used for classifying the PpdD pili segments into different groups using a multi-reference alignment. (C,D) Fourier Shell Correlation (FSC) curves for the PpdD pili reconstruction. (C) The FSC of two half-maps shows a resolution of 8 Å at FSC=0.143. (D) The FSC curve for the refined PpdD pili model and the PpdD density map shows a resolution of 8.5 Å at FSC=0.38 ( $=\sqrt{0.143}$ ).

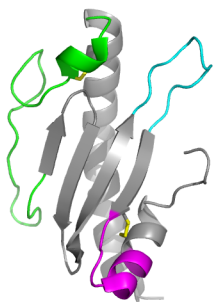


**Figure S4; Related to Figure 3: EHEC PpdD pilus contact map and evolutionary contact predictions.** Inter-residue contacts in PpdD pilus ( $d_{ij} < 8 \text{ \AA}$ ) are shown in grey (intra-protomer) and pink (inter-protomer). High confidence evolutionary contact predictions from Gremlin (Kamisetty et al., 2013) are shown in green (consistent with PpdD pilus model with  $d_{ij} < 10 \text{ \AA}$ ) and red (inconsistent).

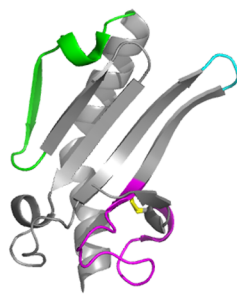


**Figure S5; Related to Figure 6: Buried surface areas of inter-subunit interfaces in T4Pa structures.** Surface area was determined using atomic solvent accessible areas of isolated and complexed pilin subunits computed with the NACCESS program (Hubbard and Thornton, 1993).

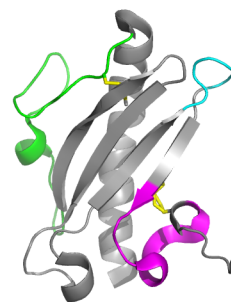
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EHEC PpdD



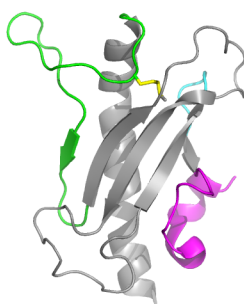
*F. tularensis* PilE  
(3SOJ)



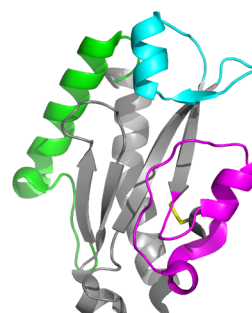
*P. aeruginosa* K122-4 PilA  
(1QVE)



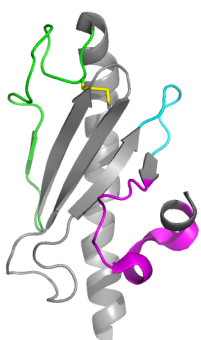
*P. aeruginosa* K PilA  
(1OQW)



*D. nodosus* FimA  
(3SOK)



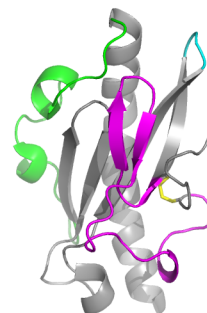
*P. aeruginosa* Pa110594 pilA  
(3JYZ)



*A. baumannii* PilA  
(5IHJ)

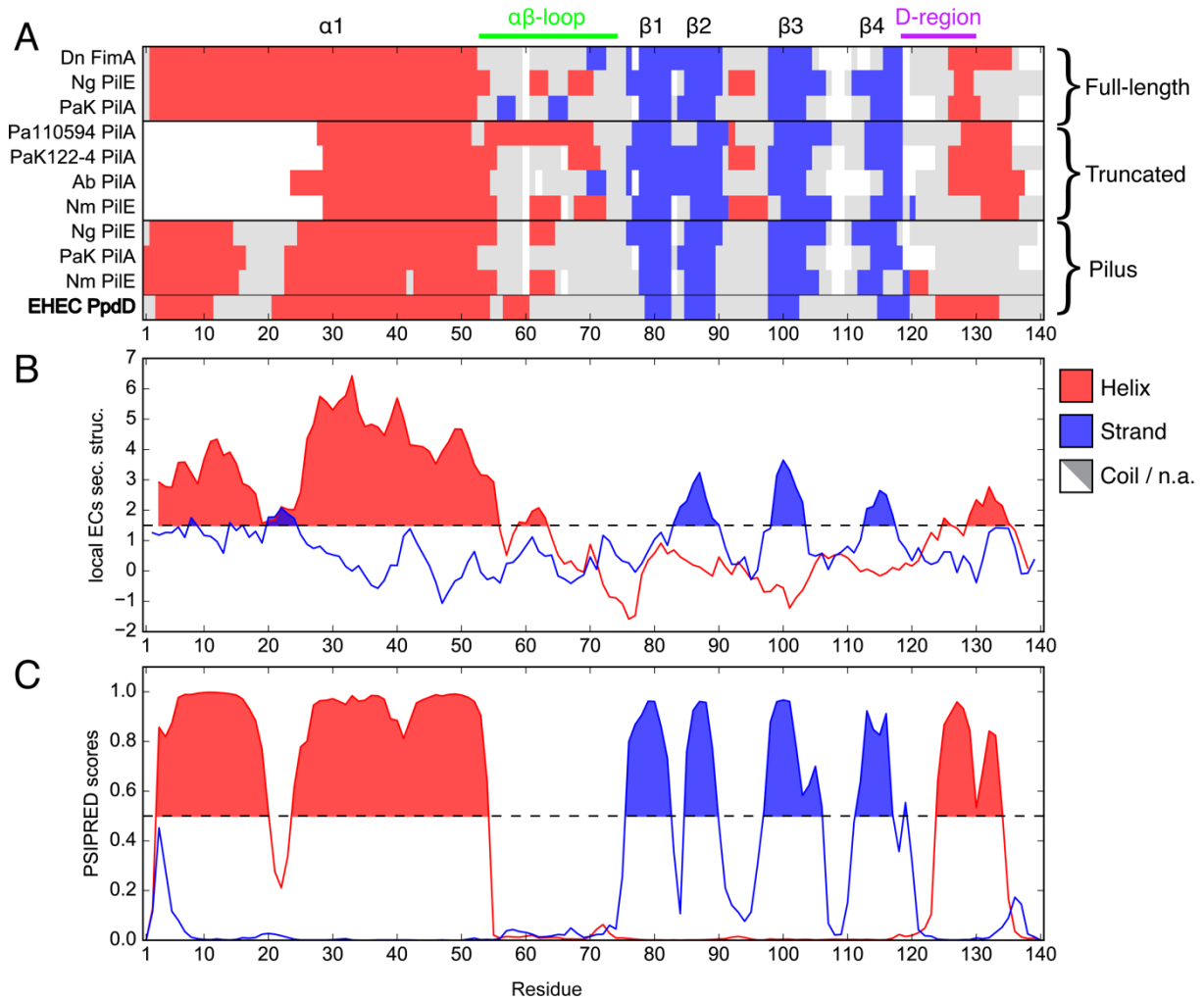


*N. meningitidis* PilE  
(5JW8)

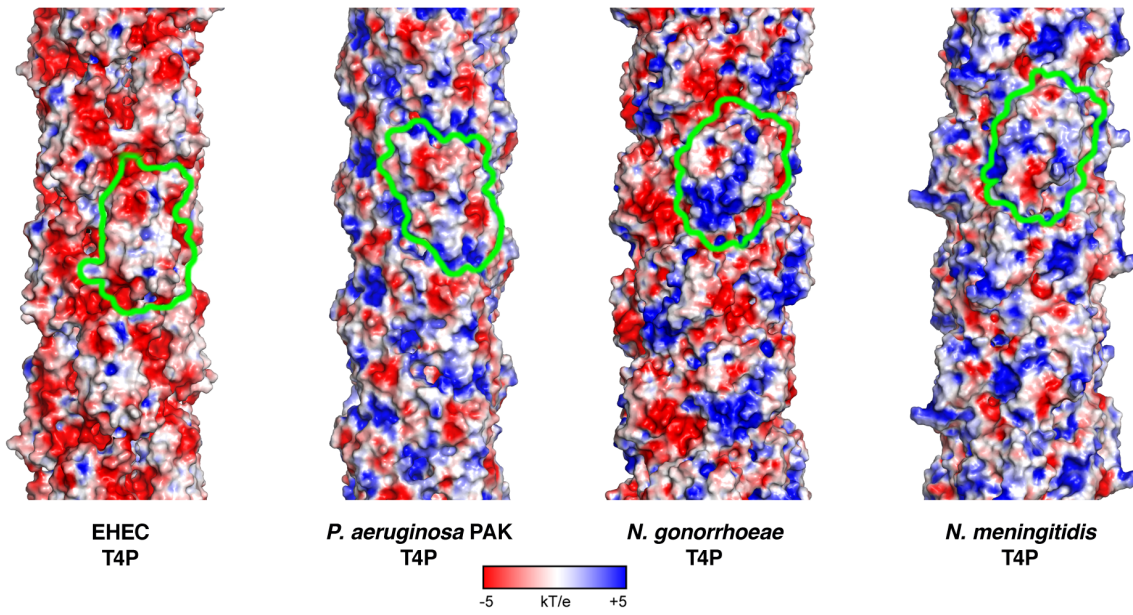


*N. gonorrhoeae* PilE  
(1AY2)

**Figure S6; Related to Figure 1: Structural comparison of major T4a pilins (periplasmic domains).  $\alpha/\beta$  loop is shown in green,  $\beta_3/\beta_4$  loop in cyan and D-region in magenta.**



**Figure S7; Related to Figure 7: Secondary structure elements in T4a pilins.** (A) Position of secondary structures elements of T4a pilin structures aligned on EHEC PpdD sequence and grouped according to the constructs and techniques used to solve the structure (Structures of full-length pilins solved by X-ray, truncated pilins missing the transmembrane segment and pilus structures solved by cryoEM). (B) Secondary structure score predicted from local evolutionary contacts within the T4a pilin family (Toth-Petroczy et al., 2016). (C) Secondary structure confidence score predicted with PSIPRED (Jones, 1999) on the EHEC PpdD pilin sequence.



23 **Figure S8; Related to Figure 7; Surface electrostatics potential of T4P pilus structure.**  
 24 Electrostatics potential calculations were performed using APBS (Baker et al., 2001). The  
 25 green lines delineate the surface of a single pilin subunit.  
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## Supplementary references

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3 Baker, N., Sept, D., Joseph, S., Holst, M., and McCammon, J. (2001). Electrostatics of  
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