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Minor pseudopilin self-assembly primes type II secretion pseudopilus elongation

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Running title: Role of minor pseudopilins in type II secretion

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15 **Abstract**

16

17 In Gram-negative bacteria, type II secretion systems (T2SS) assemble inner membrane
18 proteins of the major pseudopilin PulG (GspG) family into periplasmic filaments, which
19 could drive protein secretion in a piston-like manner. Three minor pseudopilins PulI, PulJ and
20 PulK are essential for protein secretion in the *Klebsiella oxytoca* T2SS, but their molecular
21 function is unknown. Here we demonstrate that together these proteins prime the pseudopilus
22 assembly, without actively controlling its length or the secretin channel opening. Using
23 molecular dynamics, bacterial two-hybrid assays, cysteine cross-linking and functional
24 analysis, we show that PulI and PulJ nucleate the filament assembly by forming a staggered
25 complex in the plasma membrane. Binding of PulK to this complex results in its partial
26 extraction from the membrane and in a 1-nm shift between their transmembrane segments,
27 equivalent to the major pseudopilin register in the assembled PulG filament. This promotes
28 fully efficient pseudopilus assembly and protein secretion. Therefore, we propose that PulI,
29 PulJ, and PulK self-assembly is thermodynamically coupled to the initiation of pseudopilus
30 assembly, possibly setting the assembly machinery in motion.

31

32 **Keywords:** bacterial protein secretion/ membrane proteins/ molecular dynamics/ pilus
33 assembly/type IV pili

Introduction

Many Gram-negative bacteria use the type II (T2SS) secretion system to secrete folded proteins from the periplasm. Human pathogens like *Pseudomonas aeruginosa*, *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) or plant pathogens like *Dickeya dadantii* use T2SS to secrete toxins and enzymes that damage specific tissues (Cianciotto, 2005; Sandkvist, 2001). The T2SS machinery is related to the systems involved in bacterial natural transformation (Chen *et al*, 2005) and in the assembly of type IV pili (T4P) and archaeal flagella (Albers and Pohlschroder, 2009; Pelicic, 2008). All these systems assemble small membrane proteins called pilins into filamentous structures, with the help of a conserved machinery localized in the plasma membrane.

All T2SSs contains five pilins (called PulGHIJK in the *Klebsiella oxytoca* Pul secreton (d'Enfert *et al*, 1987) that have been designated pseudopilins because they do not normally appear on the surface of bacteria. Based on their structural similarities with T4P, it has been postulated that T2SSs assemble periplasmic filaments called pseudopili that promote specific protein transport through the outer membrane (Pugsley, 1993b). In support of this model, the most abundant (major) pseudopilin PulG is assembled into long, surface-exposed pili when overproduced (Sauvonnet *et al*, 2000). Although necessary, PulG pilus assembly is not sufficient for secretion of the enzyme pullulanase (PulA), the specific substrate of the Pul secreton. Four low abundance (minor) pseudopilins PulH, PulI, PulJ and PulK, are essential for PulA secretion, although they have not been found in the surface-assembled pili (Sauvonnet *et al*, 2000; Vignon *et al*, 2003).

T2SS and T4P pilins are inner membrane proteins composed of a long sigmoidal alpha helical stem that includes a conserved transmembrane (TM) segment, followed by a variable globular periplasmic domain (Craig and Li, 2008; Hansen and Forest, 2006). Upon membrane insertion *via* the SRP/Sec pathways (Francetic *et al*, 2007), a positively charged peptide is removed from the N-terminus of the pilin signal-anchor by the prepilin peptidase PulO. Recently we determined the structure of the PulG pilus at pseudo-atomic resolution using a combination of molecular dynamics-based modeling and biochemical validation (Campos *et al*, 2010). We showed that pilus assembly, essential for protein secretion, involves specific electrostatic and hydrophobic contacts between neighboring pseudopilin subunits. The PulG subunits in the pilus are arranged in a right-handed helix, consistent with the structure of a complex composed of soluble domains of homologous minor pseudopilins GspI, GspJ and GspK from ETEC (Korotkov and Hol, 2008). In this structure, the three pseudopilins are

arranged in a right-handed quasi-helix, where GspK (the largest of the minor pseudopilins) caps this complex, suggesting that minor pseudopilins may localize at the tip of the filament.

Many of the T2SS components are similar to those required for T4P assembly (Ayers *et al*, 2010; Peabody *et al*, 2003). In the Pul secreton, these components include an assembly platform (Py *et al*, 2001) composed of a hexameric cytoplasmic ATPase (PulE), inner membrane proteins PulL, PulM and PulF, and an outer membrane channel formed by the secretin PulD, allowing the translocation of the protein substrate. Current models in both T4P and T2SS propose that pili are assembled from the base in the inner membrane. Successive conformational changes of the ATPase would power the membrane assembly platform components to catalyze the addition of pilin monomers, elongating the fiber (Campos *et al*, 2010; Craig *et al*, 2006; Misic *et al*, 2010).

Previous studies have suggested different functions for the components of the pseudopilus “tip complex”, including initiation and arrest of pseudopilus polymerization (Sauvonnet *et al*, 2000; Vignon *et al*, 2003), its degradation (Durand *et al*, 2005) or the opening of the secretin channel (Forest, 2008; Korotkov and Hol, 2008). To understand the molecular role of minor pseudopilins, we studied their function in the T2SS of *K. oxytoca*, functionally reconstituted in *E. coli*. Our results provide *in vivo*, *in vitro* and *in silico* evidence that pseudopilins PulI and PulJ form a complex in the membrane, to which PulK binds to form a pre-assembled pseudopilus tip. This complex “primes” the initiation of pseudopilus assembly by means of thermodynamic coupling.

Results

Defective pilus assembly in pulI, pulJ and pulK single mutants

Four minor pseudopilins PulH, PulI, PulJ and PulK, are required for efficient pullulanase secretion by the *K. oxytoca* type II secretion system (Possot *et al*, 2000). When the genes encoding the Pul secreton are overexpressed in *E. coli*, the bacteria grown on plates assemble pili on their surface, composed mainly of the major subunit, PulG (Kohler *et al*, 2004; Sauvonnet *et al*, 2000). To examine the function of minor pseudopilins in pilus assembly, we tested the effect of single pseudopilin gene deletions in *E. coli* expressing the *pul* genes in plasmid pCHAP231 (d'Enfert *et al*, 1987). Pilus assembly in these bacteria was assessed using immuno-detection of the major pilin PulG in bacterial cell- and sheared pili fractions (Fig. 1A) and the percentage of sheared PulG was quantified (Fig. 1B). The absence of PulH did not have a marked effect on the levels of PulG detected in the sheared fraction (Fig. 1A, lane

8 and Fig. 1B). In contrast, in the *pull*, *pulJ* and *pulK* mutants the amount of PulG in the sheared fraction was reduced (Fig. 1A, lane 10, 12 and 14 and Fig. 1B).

To better characterize the phenotypes of minor pseudopilin mutants, we examined the morphology of surface pili by immunofluorescence (IF) microscopy (Fig. 1C). Pili in each micrograph were counted and normalized to the number of bacteria (Fig. 1D). While the $\Delta pulH$ mutant was virtually indistinguishable from WT (Fig. 1C-D), the $\Delta pulJ$ and $\Delta pulK$ mutants had fewer pili (Fig. 1C-D). Consistent with the biochemical fractionation results, the strongest defect was observed in the $\Delta pull$ mutant, which assembled very few pili (Fig. 1C-D). Overall, the phenotypes of *pull*, *pulJ* and *pulK* mutants were highly similar, producing a reduced number of filaments. Interestingly, pili produced by the most defective mutant, $\Delta pull$, were slightly longer than those assembled by the WT (Supplementary Fig. S1A). To test whether producing PulG in excess would rescue the piliation defect in these strains, we introduced a second plasmid carrying *pulG* under *p_{lacZ}* control. This led to the assembly of longer pili in *pulJ*, *pulK*, and *pull* mutants compared to WT, but the number of pili remained reduced (Supplementary Fig. S1D-I). We conclude that pilus assembly initiation is defective in these mutants, whereas the pilus length appears to be the function of the cellular levels of PulG. All these results suggest a functional interaction between PulI, PulJ and PulK, which is required for efficient pilus assembly.

We also tested secretion of a nonacylated PulA variant in these mutants (Francetic and Pugsley, 2005). PulA secretion was abolished in all pseudopilin mutants, with the exception of *pulH*, which showed a mild defect (Fig. 1E-F), consistent with previously published data (Possot *et al*, 2000). However, PulH was required for PulA secretion when the copy number of the plasmid carrying the *pul* genes was reduced by a *pcnB* mutation (Lopilato *et al*, 1986; Vignon *et al*, 2003) (Supplementary Fig. S1B-C). These results demonstrate a strong correlation between the requirement of the minor pseudopilins PulI, PulJ and PulK for efficient pseudopilus assembly and for PulA secretion.

PulI and PulJ restore pilus assembly in a $\Delta pulHIJK$ mutant

Biochemical *in vitro* studies in *P. aeruginosa* showed that periplasmic domains of all minor pseudopilins, including the PulH homologue XcpU form a quaternary complex (Douzi *et al*, 2009). To analyze *in vivo* the function of minor pseudopilins in pilus assembly, we deleted the genes encoding all minor pseudopilins in plasmid pCHAP231 encoding the *pul* secretion, giving plasmid pCHAP8296 ($\Delta pulHIJK$). *E. coli* carrying this construct had no PulG in the sheared fraction (Fig. 2C, lane 12). Expression of minor pseudopilin genes *pulHIJK* *in trans*

under *p_{lacZ}* control (Fig. 2C, lane 2) restored pilus assembly. Although the Δ *pulHIJK* mutant and the complemented strain produced less PulG compared to WT (compare Fig. 2C, lanes 1 and 13), quantification of the percentage of sheared PulG showed similar pilus assembly efficiencies (Fig. 2D). Surprisingly, further analysis of the Δ *pulHIJK* mutant by IF microscopy revealed the presence of few pili (Fig. 2A, Δ *pulHIJK* + Vector), which appeared at least as long as pili assembled by the WT and complemented strains (Fig. 2A). This further shows that minor pseudopilins are required for efficient pilus initiation, but not for its elongation. Similar results were obtained in *E. coli* expressing *pul* genes with a Δ *pulGHIJK* mutation complemented with either *pulG* or *pulGHIJK* genes under *p_{lacZ}* control (Supplementary Fig. S2).

Despite its major importance, PulI alone was not sufficient to restore pilus assembly in the Δ *pulHIJK* mutant (Supplementary Fig. S2C-D). Therefore, we tested whether pairs of minor pseudopilins (PulH-PulI, PulI-PulJ, PulI-PulK or PulH-PulJ) could rescue the defect. While the PulH-PulJ, PulH-PulI and PulI-PulK pairs were unable to restore piliation (Fig. 2C, lanes 4, 6 and 10), PulI and PulJ together promoted efficient pilus assembly (Fig. 2C, lane 8). Interestingly, analysis of 10-fold concentrated samples showed traces of PulG in the Δ *pulHIJK* mutant complemented with *pulIK* (Fig. 2C, lane 10, and Fig. 2D). Consistent with this, IF images of the Δ *pulHIJK* mutant complemented with *pulIJ* showed ~50% of pili compared to the *pulHIJK*-complemented strain, while co-producing PulI and PulK led to only small numbers of PulG pili (Fig. 2A-B).

The minor pseudopilins do not open the secretin channel

To exclude the possibility that the defect of the Δ *pulHIJK* mutant was due to inability to open the outer membrane secretin channel (PulD), we examined by IF sphaeroplasts of *E. coli* transformed with pCHAP231 derivatives carrying *pulD*, *pulHIJK* and *pulD/pulHIJK* knockouts. As expected, IF images of intact bacteria showed no pili on the surface of the Δ *pulD* mutant (Fig. 3A, left pannel), while PulG filaments were observed in the sphaeroplasts (Fig. 3A right, arrows). However, neither Δ *pulHIJK* nor the Δ *pulD/\Delta*pulHIJK* mutant showed any such filaments, indicating that PulG pili do not assemble in the periplasm in the absence of minor pseudopilins (Fig. 3B-C). The co-expression of *pulHIJK* *in trans* from a *p_{lacZ}* promoter restored the pilus assembly in the periplasm (Fig. 3D). Taken together, our results suggest that the initiation of PulG filament assembly is impaired in the Δ *pulHIJK* mutant, but neither its elongation nor the secretin channel opening.*

The minor pseudopilins acquire a preassembled state in the membrane

We hypothesized that the ability of PulI and PulJ to promote pilus assembly was related to the binding of their globular domains (Korotkov and Hol, 2008). If this were the case, the interaction between PulI and PulJ should occur in their membrane embedded, unassembled state. To determine whether full-length PulI and PulJ interact *in vivo*, we used the bacterial two-hybrid assay that allows one to study interactions between membrane proteins (Karimova *et al*, 1998). As bait, we used PulI, which has a central position in the minor pseudopilin complex (Korotkov and Hol, 2008, Douzi, 2009 #3). We fused the T18 fragment of the *Bordetella pertussis* adenylate cyclase toxin (CyaA) to the N-terminus of the mature PulI (Fig. 4A). The T25 fragment of CyaA was fused to the N-termini of mature PulH, PulI, PulJ and PulK. The interaction of the two CyaA fragments led to the production of cAMP that was monitored by measuring the β -galactosidase activity in the *E. coli cya* mutant strain DHT1. Among the minor pseudopilins, only PulJ interacted with PulI (Fig. 4A), as indicated by the high β -galactosidase activity in *E. coli* DHT1 producing T18-PulI and T25-PulJ, comparable to that of the positive control (the yeast protein GCN4 leucine zipper region Zip fused to both T18 and T25). The β -galactosidase activities in strains producing T25-PulH or T25-PulI together with T18-PulI were similar to the negative control (T18 or T18-PulI combined with T25). The activity of the pair T25-PulK and T18-PulI appeared only slightly higher than the negative control. Because strain DHT1 does not contain a functional T2SS, our results show that PulI and PulJ interact in the inner membrane in the absence of other components of the Pul secretion.

To gain insight into the conformation of the PulI and PulJ complex in the membrane, we introduced single cysteine substitutions in the TM segments of the two proteins. In a previous study of PulG pilus assembly, we showed that double cysteine substitutions at positions 10 and 16 of PulG led to the formation of covalently cross-linked PulG multimers in the pilus fractions (Campos *et al*, 2010). Therefore, we introduced single cysteine substitutions at and near positions 10 and 16 in PulI and PulJ, respectively. *E. coli* co-producing single cysteine variants of PulI and PulJ were chemically oxidized using copper phenanthroline. To detect PulJ, we introduced a hexa-histidine tag at its C-terminus and analyzed oxidized total cell extracts using His-probe-HRP. Bacteria co-producing single cysteine substituted variant PulJL16C-His₆ together with variants PulIL10C or PulIV11C formed membrane-associated (Supplementary Fig. S3C) cross-linked products, migrating with an apparent mass of 35 kDa, corresponding to that of a PulI-PulJ heterodimer (Fig. 4B, lanes 4-5). This was confirmed by analyzing these samples with an anti-PulI antibody (Fig.

4C, lanes 4-5). However, variants PulIV8C, PulIA9C, and PulIV12C formed only homodimers (expected mass, 26 kDa) (Fig. 4C lanes 2, 3 and 6). Homodimers also formed when PulI variants were co-produced with PulJA15C-His₆, but cross-linked heterodimers between PulI and PulJ were not observed (Supplementary Fig. S3A). This result indicates that the formation of cross-linked heterodimers correlates uniquely with the positions of the cysteine-substituted residues in PulI and PulJ and not with the fact that variants like PulIV8C form homodimers. Similar cross-linking pattern of PulI cysteine-substituted variants with PulJL16C-His₆ was observed in the presence of the *pul* genes with a *pulHIJK* deletion (Supplementary Fig. S3B). Interestingly, after only 10 minutes of oxidation, variant PulJL16C-His₆ formed heterodimers mainly with PulIL10C (Supplementary Fig. S3D), suggesting a closer proximity of this residue with PulJ L16. All of these results indicate that, if initially the TM segments of monomeric PulI and PulJ reside at similar positions along the membrane plane, binding of PulI to PulJ induces a conformational change, shifting their TM segments by approximately 1 nm. This shift between the interacting residues of PulI and PulJ in the membrane is identical to the 1-nm axial rise between major pseudopilins in the assembled PulG filament (Campos *et al*, 2010). Therefore, we propose that the minor pseudopilins rearrange in the membrane to form a pre-assembled pseudopilus.

Despite the fact that significant interactions were not observed between PulI and PulK in two-hybrid experiments, some PulG pili were assembled in the Δ *pulHIJK* mutant complemented with *pulIK*, indicating a weak productive interaction. To test whether these proteins interact in a similar fashion as PulI and PulJ, we introduced single cysteine substitutions at and near positions 16 and 10 in PulI and PulK, respectively. Immunoblot analysis of oxidized *E. coli* co-producing PulIA16C and PulK cysteine-substituted variants showed that PulKL10C and PulKA11C, but not PulKI9C, cross-linked to PulIA16C, as shown by the presence of a band with an apparent mass corresponding to a PulI-PulK complex (48 kDa) (Fig. 4D-E, arrows). Therefore, PulI and PulK do indeed interact in the membrane, and like PulI and PulJ, they might rearrange upon binding to form a staggered array, with a shift corresponding to the axial rise in the PulG filament.

To determine if the full-length minor pseudopilins form a tripartite complex, we performed a pull-down experiment using a hexahistidine tagged PulK variant in detergent-solubilized membranes. *E. coli* producing a plasmid-encoded prepilin peptidase PulO (to ensure pseudopilin processing) was transformed with a plasmid encoding WT versions of all pseudopilin genes and PulK-His₆, or plasmids encoding variants PulIL10C, PulJL16C and PulK-His₆ instead of PulI, PulJ and PulK respectively. As a control, we used a plasmid

encoding PulIL10C, PulJL16C and WT PulK. Although some non-specific binding of PulK was observed to Ni-IDA magnetic beads (Fig. 4F lane 5), PulK-His₆ strongly bound to the beads, and the Pull-PulJ cross-linked product appeared specifically in the elution fraction (Fig. 4F lane 6). This result suggests that the interaction of Pull, PulJ and PulK resisted membrane solubilization. Non cross-linked Pull also eluted with PulK-His₆ (Fig. 4F lane 4), although, for lack of specific antibodies, we could not determine whether PulJ was part of this complex. All of these results suggest that the minor pseudopilins interact in the membrane to form a pre-assembled pseudopilus in the absence of other T2SS components.

Molecular dynamics of full-length minor pseudopilin complexes

To analyze how Pull, PulJ and PulK might interact in the membrane, we modeled TM segments onto the crystallographic structure of ETEC GspJ-GspI-GspK complex, which showed a quasi-helical register, as reported (Korotkov and Hol, 2008). When introduced into a palmitoyl-oleoyl ethanolamine (POPE) model membrane, their N-termini were placed at different levels of the membrane plane (Fig. 5A, arrows). Next, we removed for each protein its two partners and performed molecular dynamics (MD) simulations (Supplementary Video S1). After 20 ns of simulation, all three proteins underwent substantial lateral diffusion and their N-termini adopted similar positions in the membrane plane (Fig. 5B-C, arrows). The mean position of each residue, with respect to the membrane glycerol backbone atoms in the last 5 ns (Fig. 5E) showed that the three proteins reintegrated to a similar extent into the POPE bilayer. These positions could correspond to their initial state upon membrane insertion and maturation, prior to the complex formation.

We also performed an MD simulation of the three proteins together (Supplementary Video S2). This complex was stable (Supplementary Fig. S4) and the mean position of TM segment residues in the membrane showed that GspI and GspJ are completely embedded (Fig. 5E-F). However, GspK TM segment remained partially extracted, with a displacement of ~1nm with respect to GspI and GspJ (Fig. 5F). This displacement created a severe membrane deformation (Fig. 5J). Nevertheless, the 1-nm helical register imposed by the GspI-GspJ-GspK soluble domains was maintained inside the membrane. This is shown in the contact map of GspI and GspJ or GspK where neighboring residues are shifted from the diagonal, unlike those of GspI with itself (Fig. 5G). Interestingly, the main interactions between Pull and PulJ or PulK shown by cross-linking were represented in these maps (Fig. 5I).

We showed that Pull and PulJ promote PulG pilus assembly and that they interact in the membrane. To examine this interaction, we performed MD simulations of the GspI-GspJ

complex with modeled TM segments (Supplementary Video S3). This complex was stable (Supplementary Fig. S4) and, as in the GspI-GspJ-GspK simulation, the TM segments remained staggered. In the contact map of GspI and GspJ neighboring residues are shifted from the diagonal (Fig. 5H). However, only some of the interactions between Pull and PulJ tested by cross-linking were present (Fig. 5I).

Next, we clustered by similarity all MD simulation-derived structures by aligning their periplasmic α -helical domains and observing the differences in their TM segments. In the representative structures of the main clusters from GspI-GspJ-GspK and two GspI-GspJ simulations, GspJ is curved around glycine 36 (Fig. 6A). In contrast, this bending was not present in the main clusters derived from the GspJ alone simulation (Fig. 6B). This suggests that this conformational change occurs in the presence of GspI and GspK. GspI and GspK also showed some bending (Fig. 6A), although some of the conformations found in the dimer and trimer MD simulations also occurred in the main clusters of the GspI and GspK alone simulations (Fig. 6C), suggesting some intrinsic flexibility. One interesting feature is that in both simulations the GspI-GspJ dimer tilted in the membrane (Fig. 6D). Apparently, the hydrophobic TM segments maximized their interaction with the phospholipid fatty-acyl chains, bringing the GspI β 3- β 4 loop close to the phospholipid headgroups. This induced tilting could favor the interaction with GspK. Interestingly, a similar behavior has been observed for *P. aeruginosa* PilA (Lemkul and Bevan, 2011).

Together, these *in silico* results show that: (1) the helical register expected for the periplasmic assembled pseudopilus is maintained in the membrane embedded GspI-GspK-GspJ complex, (2) that GspJ undergoes conformational changes when bound to GspI and GspK and (3) that GspK remains partially extracted from the membrane in this complex, inducing a local membrane deformation.

Pull and PulJ couple their binding energy to pilus assembly initiation

We have shown so far that there is a correlation between the binding of Pull and PulJ and the ability of this complex to restore piliation in the *ApulHIJK* mutant. To examine this correlation, we designed six mutations in *pull* that could disrupt Pull binding to PulJ and we tested their impact both on this interaction and on their ability to restore efficient pilus assembly when co-produced with PulJ in the *ApulHIJK* mutant. The invariant residue E5 of PulG, essential for pilus assembly (Pugsley, 1993a), is also conserved in Pull. However, when substitution E5K was introduced into T18-Pull, its interaction with T25-PulJ was comparable to WT (Fig. 7B). Consistent with this result, the double mutant Pull-L10C/E5K cross-linked

to PulJ-L16C (Fig. 7C lane 2). Residue E35 (Fig. 7A) lies close to the interface of GspI and GspJ, but as the substitution E35K in PullI affected protein stability (Fig. 7C, lane 3) this variant was not analyzed further. The GspI residue W42 (Fig. 7A) hides ~38 Å² of accessible surface upon GspJ and GspK binding. T18-PullW42A interacted comparably to WT in the two-hybrid assay (Fig. 7B), but the double mutant PullI-L10C/W42A showed only traces of the PulJ-L16C cross-linked product (Fig. 7C, lane 4, arrow). Residue E45 in ETEC GspI lies in the interface between GspI, GspJ and GspK (Fig. 7A). The substitution of its equivalent (D45K) in T18-PullI had no effect in the two-hybrid assay (Fig. 7B), and the double mutant PullI-L10C/D45K cross-linked to PulJ-L16C (Fig. 7C, lane 5). Residue N46 (Fig. 7A) is highly conserved and is involved in many important contacts with GspJ (Korotkov and Hol, 2008). Substitution N46K in T18-PullI abolished binding to T25-PulJ in the two-hybrid assay (Fig. 7B), and the variant PullI-L10C/N46K did not cross-link to PulJ-L16C (Fig. 7C, lane 6). Furthermore, PullI-N46K did not restore efficient pilus assembly in the *ΔpulHIJK* mutant when co-produced with PulJ (Fig. 7D, lane 4). Double-substituted variant PullI-W42A/N46K was similar to PullI-N46K (Fig. 7B and C, lane 7), although it initiated pilus assembly even less efficiently (Fig 7D, lane 6). All these results indicate a strong correlation between the interaction of PullI and PulJ (as measured by the two-hybrid assay and cysteine cross-linking) and pilus assembly initiation in the *ΔpulHIJK* mutant. Furthermore, they demonstrate that when PullI and PulJ binding is disrupted, the ~1 nm shift in their TM segments is also affected. This suggests that the binding energy of PullI and PulJ is necessary for the proposed conformational change that would shift their TM segments to acquire a pseudopilus-like structure in the membrane.

Surprisingly, while PullI mutant variants restored PulG pilus assembly to different extents (Supplementary Fig. S5) in the *ΔpulHIJK* mutant, they could all restore full pullulanase secretion in the *pulI* deletion mutant, even at low *pul* gene expression levels (Fig. 7F). To test whether this was due to the presence of PulK, we produced PulJ and the most defective PullI mutant variants with or without PulK in the *ΔpulHIJK* mutant. Both PullI-N46K and PullI-W42A/N46K could promote efficient pilus assembly when PulK was present (Fig. 7D-E). Presumably, PulK could promote binding of these PullI variants to PulJ to allow PulA secretion.

Discussion

We show here for the first time that the minor pseudopilins PulI, PulJ and PulK interact *in vivo* in the bacterial inner membrane to initiate PulG filament assembly. IF microscopy analysis revealed highly similar phenotypes of single *pulI*, *pulJ* or *pulK* mutants, all of which assembled reduced number of fibers on their surface compared to WT. The severe defect found in the *pulI* mutant is consistent with the central position of this protein in the complex formed by the pseudopilin globular domains *in vitro* (Douzi *et al*, 2009; Korotkov and Hol, 2008). In contrast, pilus elongation was efficient in mutants lacking PulI, PulJ, PulK, or all minor pseudopilins. The defect in assembly initiation led to the assembly of fewer and longer fibers, which was accentuated by overproducing PulG, with the *pulI* mutant making very rare and long pili (Supplementary Fig. S1 and S2). These observations are consistent with the model in which the pilus length is directly proportional to the size of the PulG pool in the membrane and inversely proportional to the number of active assembly sites (Fig. 8A-B). Once the energetic barrier required for the initiation is overcome, pilus assembly continues as a function of the major pseudopilin concentration in the membrane. Thus, in the *pulI*, *pulJ* and *pulK* mutants, the reduced initiation efficiency would lead to pseudopilin accumulation in the membrane, favoring the elongation of the few pili whose assembly had already started. Together, our results suggest that none of the minor pseudopilins plays an active role in the pseudopilus length control. Finally, the correlation between the requirement of the minor pseudopilins PulI, PulJ and PulK but not of PulH for efficient pilus assembly and protein secretion, suggests a role for the PulI-PulJ-PulK complex in initiating pseudopilus assembly during protein secretion.

The function of the minor pseudopilin PulH appears to be unrelated to the assembly initiation, since the number of pili in the *pulH* mutant is similar to WT. PulH could instead be involved in the elongation step, at least under physiological conditions where this protein is essential for secretion (Supplementary Fig. S1). This is consistent with the fact that the PulH requirement can be partially overcome by overproducing the *pul* genes. Thus, structural complementarity between the tip complex and the PulG filament is not entirely required for pseudopilus elongation. Therefore, additional factors, like the assembly machinery at the inner membrane, could act downstream of the pseudopilus priming, as discussed below.

We show here that the minor pseudopilins have no effect on the opening of the secretin channel. When the *pul* genes are overexpressed in *E. coli* grown on agar plates, the pili are surface exposed in single or multiple minor pilin mutants, and rare pili are observed even in the $\Delta pulHIJK$ mutant. This suggests that the secretin channel in the outer membrane allows the passage of fibers composed only of PulG. However, in the absence of the secretin

channel (*ΔpulD* mutant), the pseudopilus is formed in the periplasm, as observed previously (Vignon *et al.*, 2003). We found no periplasmic pili in the *ΔpulHIJK/ΔpulD* mutant, confirming the role of minor pilins in initiating the filament assembly. In addition, the expression of the minor pseudopilin genes *in trans* restored the periplasmic fiber accumulation. Therefore, the initiation of pseudopilus assembly could be the main function of the PulI-PulJ-PulK complex, although at this point we can not discard the possibility that it could participate in any event downstream of pseudopilus assembly to regulate secretion in liquid-grown bacteria, where pseudopili do not extend beyond the cell surface. Whether the tip pseudopilins are dispensable or degraded after completing pseudopilus initiation remains an open question.

Pilins are synthesized with a positively charged N-terminal signal anchor, which is cleaved at the level of the cytoplasm-membrane interface by prepilin peptidase. Presumably, the cleavage site and the resulting N-termini of the matured pilins lie at approximately the same level in the membrane plane. This is consistent with the results of the MD simulations of PulI, PulJ and PulK monomers, in which the three proteins are positioned at the same level in the absence of their binding partners (Fig. 5. B-E). However, both molecular dynamics and cysteine cross-linking suggested that the formation of the native PulI-PulJ-PulK complex in the absence of other components of the Pul secreton leads to rearrangements of their TM segments. As a result of these rearrangements, the interactions between the minor pseudopilin TM segments involve residues that have equivalents in the filament formed by the major pseudopilin PulG. Moreover, the interaction between PulI and PulJ was required for significant pilus assembly initiation in the context of the *ΔpulHIJK* mutation. Taken together, our results provide strong evidence that the formation of the PulI-PulJ complex in the membrane nucleates the pre-assembled filament-like structure (Fig. 8C). Although this complex plays a central and initial role, fully efficient pilus assembly (Fig. 1F) and protein secretion require PulK. We showed that PulK bound to the PulI-PulJ complex and that PulK binding to PulI led to the rearrangement of its TM segment. PulK binding to PulI-PulJ in the membrane would lead to their stabilization as suggested by the MD simulations of a complex composed of full-length GspI-GspJ-GspK (Supplementary Video S2). In addition, binding of PulK to PulI-PulJ may induce an upward movement, causing a partial extraction of the PulK TM segment to acquire a pseudopilus-like structure in the membrane (Fig. 8D). The tilting of PulI and PulJ complex in the membrane, suggested by the molecular dynamics, might facilitate this binding, together with the fact that the α -helical N-terminal domain of PulK

remains straight. Furthermore, the presence of a kink in the GspJ TM segment is a structural similarity with major pseudopilins, where the highly conserved kink in the α -helical N-terminal domain centered at residue P22 is necessary for efficient assembly of PulG into pili (Campos *et al*, 2010).

If one considers pseudopilus assembly as a biochemical reaction, the binding of the minor pseudopilins could have three consequences: kinetic, thermodynamic and structural (or geometrical). The correlation between the binding of PulI to PulJ in the membrane and the pilus assembly supports the idea that the interaction between the minor pseudopilins reduces the kinetic barrier to initiate pseudopilus assembly. The formation of the PulI-PulJ-PulK complex could also have thermodynamic consequences, since tight binding between their globular domains could stabilize the whole pseudopilus, favoring its assembly. In this respect, it is interesting to note that GspK reduced the RMSD of GspI and GspJ in MD simulations (Supplementary Fig. S3). Finally, the minor pseudopilin complex formation may have structural consequences. Evidence for this suggestion comes from the fact that GspK maintained its helical register in the GspI-GspJ-GspK complex in MD simulations and that PulI could be cross-linked in the membrane with PulJ and PulK in a staggered manner. Furthermore, GspK binding to GspI-GspJ dimer induced a severe membrane deformation *in silico*. We propose that the energy used to deform the membrane during MD simulations is directly transduced to the assembly platform, and may lead to its activation. The fact that pilus assembly occurs spontaneously on rare occasions supports this model, suggesting that the tip complex may reduce an energy barrier for a binding event leading to the assembly initiation.

How exactly could the primed conformation of minor pseudopilins initiate the pilus assembly? Although structural information is available for the soluble domains of homologues of PulL, PulF and two domains of the ATPase PulE, the organization of these proteins in the membrane remains unclear, and their interactions with pseudopilins are largely unexplored. Recent studies in *V. cholerae* show that EpsL interacts with the major pseudopilin EpsG, providing the first direct link between pseudopilins and the assembly platform (Gray *et al*, 2011). Since EpsE interaction with the membrane phospholipids is required for efficient ATP hydrolysis (Camberg *et al*, 2007), one plausible model is that the upward movement induced by the minor pseudopilin complex formation is transduced *via* PulL or PulF to PulE, bringing it close to the membrane to activate ATP hydrolysis. This cascade of conformational changes would set the assembly platform in motion by coordinated ATP hydrolysis, leading to insertion of PulH and PulG into the growing pseudopilus (Fig.

8E). Interestingly, the T4P retraction ATPase PilT (Misic *et al*, 2010) has three main conformations (ready, active and release). If the action of PilT is similar (or the reverse) to that of PulE, one could speculate that the minor pseudopilins symmetry may correspond to, fit, or lead to these three states.

Type 4a pilus assembly systems contain a set of pilin genes arranged in a similar manner as the *pulHIJK* genes, and certain features suggest that they share a common function (Forest, 2008; Korotkov and Hol, 2008). First, some of them encode a larger protein (equivalent to PulK) that could cap the tip of the pilus. Second, this protein does not have the conserved residue E5, which would be dispensable at the tip, as it is in PulK (Vignon *et al*, 2003) (Supplementary Fig. S6). Third, genetic evidence suggests that in T4P, these minor pilins "counteract" retraction because the pilus assembly defects of single deletion mutants in these genes are suppressed by a deletion of the gene encoding the retraction ATPase PilT (Carbonnelle *et al*, 2006; Giltner *et al*, 2010; Winther-Larsen *et al*, 2005). Therefore, these minor pilins could form a complex that would shift the equilibrium towards pilus assembly by acquiring a pre-assembled conformation. It is also tempting to speculate that their interaction could participate in the switch between assembly and disassembly (Maier *et al*, 2004), especially if minor pilins were not located exclusively at the tip (Giltner *et al*, 2010). Therefore, minor pilin binding coupled to partial extraction of TM segments could represent a general mechanism in the initial events of pilus assembly in T4P and other related systems such as DNA competence (Chen *et al*, 2005), archaeal pili and flagella (Albers and Pohlschroder, 2009).

In conclusion, we propose that the intrinsic binding properties of the minor pseudopilins PulI, PulJ and PulK lead to their self-assembly into a pseudopilus-like complex in the membrane. This event could in turn induce and coordinate key conformational changes of the membrane assembly platform, resulting in its activation to initiate pseudopilus polymerization. The exact sequence and molecular details of this process will be a subject of future studies.

Materials and methods

Bacterial strains, plasmids, and molecular biology techniques

The *E. coli* K12 strains PAP7460 (Possot *et al*, 2000) and PAP5207 (Campos *et al*, 2010) were used in this study. Plasmids used in this study are listed in Supplementary Table S1. DNA extraction, plasmid constructions and DNA transformation were performed as described

(Maniatis *et al*, 1982). Site-directed mutagenesis was performed using a modified Quick change method and the Pwo DNA polymerase (Roche). Oligonucleotides, listed in Supplementary Table S2, were synthesized by Sigma Genosys. All plasmids were sequenced by GATC.

Pilus assembly, shearing and fractionation

Shearing assays was performed as described (Sauvonnet *et al*, 2000). Bacteria carrying derivatives of the plasmid pCHAP231, which contains all the *pul* genes (Table S1) were grown overnight on LB agar containing 100 g/ml of ampicillin (Ap), 25 g/ml chloramphenicol (Cm) and 0.4 % maltose to induce the expression of the *pul* genes. Bacteria were collected and resuspended in LB medium at 10 OD_{600nm}. mL⁻¹ and vortexed (sheared) for 2 minutes to release pili into the medium. Bacteria were centrifuged at 16 000 g in a table-top centrifuge for 5 minutes, the pellet was collected and resuspended in the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (cell fraction). The sheared fraction was further centrifuged for 15 minutes to remove residual bacteria, the pellet was discarded and the supernatant was precipitated with 10% trichloroacetic acid. After centrifugation for 15 min at 16 000 g, the pellet was washed twice in acetone, air-dried and resuspended in SDS-PAGE sample buffer.

Pullulanase secretion assay

Plasmid pCHAP231 carrying the gene encoding a non-acylated variant of PulA pCHAP8185 and its mutant derivatives (Supplementary Table S1) were introduced in strain PAP7460 or its *pcnB::Tn10* derivative PAP5207. Bacteria were grown to late exponential phase in LB media containing appropriate antibiotics and 0.4 % maltose. For the *pcnB* strain, media were supplemented with 1 mM IPTG. Cultures were normalized to OD_{600nm} of 1 and centrifuged for 5 min at 16 000 g. Bacterial pellets was resuspended in the initial volume of Laemmli sample buffer. The supernatant fractions were transferred to fresh tubes and centrifuged for another 10 min. Samples were taken off top and mixed with an equal volume of 2 x Laemmli sample buffer. The equivalent of 0.05 OD_{600nm} of cell and supernatant fractions was analyzed by SDS-PAGE and immuno-detection.

Immunodetection

Immunoblotting was performed as described (Sauvonnet *et al*, 2000). Proteins were separated by SDS-PAGE in tricine (Schagger and von Jagow, 1987) gels containing 10-15%

acrylamide, transferred onto nitrocellulose membranes (Amersham ECL) using semi-dry electro-transfer. Membranes were blocked with 5% milk in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) and incubated in the specific antiserum (anti-PulG at 1/2000, anti-PulI at 1/500, anti-PulK at 1/1000, anti-PulA 1/2000 or anti-LamB 1/2000) followed by horseradish peroxidase-coupled anti-rabbit antibody (1/40000; Amersham). Membranes were developed by enhanced chemiluminescence ECL-plus (GE-healthcare) and recorded using the STORM phosphoimager (Molecular Dynamics). For detection of proteins containing a hexahistidine tag, nitrocellulose membranes were blocked with 1% BSA in TBST and incubated with a dilution of 1/2000 of His-Probe-HRP (Thermo Scientific). Membranes were developed using West Pico Chemiluminescent Substrate (Thermo Scientific). Anti-PulI and anti-PulK peptide antibodies were produced and purified by Genscript (USA).

Fluorescence microscopy

Immunofluorescence (IF) labeling of pili was performed as described (Vignon *et al*, 2003). Bacteria grown in the same conditions as for pilus assembly assay, were gently resuspended and directly immobilized on poly-L-lysine coated coverslips. Samples were fixed for 20 minutes on 3.7% formaldehyde, blocked with 1% BSA in PBS and incubated with anti-PulG antibodies (1:2,000) and secondary Alexa Fluor 488- coupled anti-rabbit IgG (Invitrogen). Samples were examined with an Axio Imager.A2 microscope (Zeiss). Images were taken with AxioVision (Zeiss) and processed in ImageJ (Abramoff *et al*, 2004).

Sphaeroplast preparation

Sphaeroplasts were prepared as described (Randall and Hardy, 1986). Bacteria were resuspended in 0.5 ml of 0.1 M Tris-acetate (pH 8.2), 0.5 M sucrose and 5 mM EDTA. Lysozyme was added (0.1 mg/ml) followed by addition of 0.5 ml of ice-cold water. After incubation for 5 min on ice, MgSO₄ was added to a final concentration of 18 mM to stabilize the sphaeroplasts. Sphaeroplasting was monitored by light microscopy to verify that bacteria became round (Birdsell and Cota-Robles, 1967). Sphaeroplasts were immobilized onto poly-lysine coverslips, washed with 0.05M Tris acetate pH8.2, 0.25 M sucrose, 10 mM MgSO₄, and fixed in 3.7% formaldehyde for IF.

Molecular dynamics simulations

To generate full-length models of GspI, GspJ and GspK, we extended the structures of their globular domains (pdb code: 3CI0) by modeling the missing N-terminal hydrophobic

segments as straight alpha helices. Simulations were performed using the GROMACS package (Hess *et al*, 2008) version 4.5 (Bjellmar *et al*, 2010). The proteins were treated using the CHARMM22/CMAP force field (MacKerell *et al*, 1998), and the lipid molecules using the new CHARMM36 parameter set (MacKerell *et al*, 2010). The dimeric and trimeric protein complexes were placed in pre-equilibrated membrane systems. Each system was equilibrated over 50 ns, followed by 50 ns production MD simulations. The monomeric proteins were placed in pre-equilibrated membrane/solvent systems so that they had the same initial position and orientation as in the trimeric complex simulation systems. Following position-restrained equilibration, 20 ns production MD simulations were carried out. Clustering analysis of each simulation was carried out with GROMACS 4.5, with a neighbor-list cut-off in the pair-wise RMS difference of 0.25 nm. Trajectories were fitted to the backbone atoms of the upper regions of each N terminal helix (residues 41-53, 36-57, and 29-58 in GspI, GspJ, and GspK, respectively). Clustering was performed on the backbone atoms of the bottom regions of each N terminal helix (residues 5-41, 5-46 and 5-29 in GspI, GspJ, and GspK, respectively). The three main clusters of the GspI-GspJ, GspI-GspJ-GspK and GspK alone were present ~50% of the time in each simulation, while the 6 main clusters of the GspI alone and GspJ alone were present 50% of the time. Detailed methods for MD simulations are shown in Supplementary material.

Bacterial two-hybrid assay

To produce hybrid proteins with N-terminal T25 and T18 fragments of *Bordetella pertussis* CyaA we used plasmids pKT25 and pUT18c (Karimova *et al*, 1998). Primers are listed in Supplementary Table S2. The *cya* mutant *E. coli* strain DHT1 was co-transformed with plasmids containing T18- and T25- chimera. Bacteria were grown for 48 hrs at 30°C on LB medium, containing 100 g/ml of ampicillin (Ap) and 25 g/ml Kanamycin (Km). Randomly picked single colonies were inoculated into LB medium and pre-cultured overnight. Cultures were diluted in medium containing 1 mM Isopropyl β -D-1-thiogalactopyranoside, 100 g/ml of ampicillin (Ap) and 25 g/ml Kanamycin (Km) for β -galactosidase assays, which were performed as described (Miller, 1972). At least three independent experiments with quadruplicates were performed for each sample.

Copper phenanthroline oxidation

Bacteria carrying cysteine-substituted variants of minor pseudopilins were grown in liquid LB media to OD₆₀₀ 1-1.5. Bacteria were harvested, washed with M63 salts (100 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 1.7 M FeSO₄·7H₂O, pH 7) (Miller, 1972) and incubated 10 minutes at 30°C to equilibrate. Copper phenanthroline was added to 1.5 mM and incubated for 10-60 minutes in M63 salts. The reactions were stopped by adding 5 mM EDTA and 25 mM N-ethylmaleimide. Bacteria were washed once in M63 salts and resuspended in SDS-PAGE sample buffer.

PulK-His pull-down

Bacteria were oxidized using copper phenanthroline for one hour and the reaction was stopped with EDTA and N-ethylmaleimide. Bacteria were broken by freeze-thaw and sonication. Non-broken bacteria were removed by centrifugation at 4000 g and membrane fractions were then prepared by centrifugation at 112000 g for 1 hour. Membranes were resuspended in 50 mM phosphate-buffer, 150 mM NaCl pH 7.4 and solubilized by the addition of 2% Triton X-100. Solubilized membranes were then incubated with nickel-IDA (Ademtech) magnetic beads for 30 minutes, washed three times in phosphate-buffer, and eluted with 0.5 M imidazol in 0.2% Triton X-100.

Supplementary information is available at *The EMBO Journal* Online.

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Author contributions: D.A.C. and O.F. designed the study and wrote the manuscript, conducted the experiments and with contributions from M.C and A.P.P. analyzed the data. P.J.B. performed molecular dynamics and analyzed the data.

Conflict of interest

The authors declare that they have no conflict of interests.

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Figure Legends

Figure 1 Assembly of PulG pili in mutants lacking single minor pseudopilins. (A) PulG immunodetection in 0.005 OD_{600nm} units of cell and sheared fractions (C, SF) of *E. coli* carrying the *pulG* gene in a plasmid (*pulG* alone), or the *pul* genes (WT) on plasmid pCHAP231 or its minor pseudopilin gene deletion derivatives. LamB porin is used as a lysis control. (B) PulG percentage in SF (mean + SD) from three independent experiments like the one in (A). N.A., not applicable. (C) Immunofluorescence (IF) and phase contrast microscopy of strains analyzed in (A). PulG staining is in green and DAPI in magenta. Scale bar, 5 μ m. (D) Box plot of normalized number of pili observed in (C) from ~40 randomly selected areas in two independent experiments. (E) PulA immunodetection in cell extracts and supernatants (C, SN) of bacteria carrying *pulA* on a plasmid (+*pulA*), or expressing all *pul* genes on plasmid pCHAP8185 (WT) or its minor pseudopilin single deletion derivatives. (F) Percentage of secreted PulA (mean + SD) from five independent experiments like the one in (E).

Figure 2 Pull and PulJ initiate pilus assembly in the Δ *pulHIJK* mutant. (A) IF and phase contrast microscopy of *E. coli* expressing the *pul* genes (WT) on a plasmid or its minor pseudopilin deletion derivative (Δ *pulHIJK*). Δ *pulHIJK* mutant complemented with empty vector or genes *pulHIJK*, *pullJ* and *pullK*. PulG staining is in green and DAPI in magenta. Scale bar, 10 μ m. (B) Box plot of normalized number of pili observed in (A) from ~40 randomly selected areas in two independent experiments. (C) PulG detection in cell and sheared fractions (C, SF) from WT and the Δ *pulHIJK* mutant complemented with empty vector or genes *pulHIJK*, *pulHJ*, *pulHI*, *pullJ*, and *pullK*. The equivalent of 0.005 OD_{600nm} units (1X) or 0.05 OD_{600nm} units (10X) of C and SF was analyzed. (D) Percentage of PulG in SF (mean + SD) from three independent experiments like the one shown in (C) with 0.05 OD_{600nm} equivalent analyzed.

Figure 3 Periplasmic assembly of PulG pili in an outer membrane secretin mutant. IF microscopy showing intact (left) and sphaeroplasted (right) *E. coli* expressing *pul* genes with (A) *pulD*, (B) *pulHIJK*, (C) *pulD/pulHIJK* mutations or (D) *pulD/pulHIJK* mutation complemented with *pulHIJK*. PulG staining is in green and DAPI in magenta. Arrows indicate PulG pili. Scale bar, 15 μ m.

Figure 4 Preassembled conformation of minor pseudopilins in the membrane. (A) Interaction of minor pseudopilins analyzed by bacterial two-hybrid assay. β -galactosidase activity (mean + SD) of DHT1 bacteria co-producing T18-PulI with T25 PulH, PulI, PulJ and PulK chimera or T25 alone. Zip-T18 and Zip-T25 chimera are used as positive control. (B-C) Position specific cysteine cross-linking of *E. coli* co-producing PulJL16C-His₆ with PulIV8C, PulIA9C, PulIL10C, PulIV11C, PulIV12C or empty vector. Cell extracts were analyzed using His-Probe-HRP (B) or anti-PulI antibody (C). (D-E) Cysteine cross-linking of bacteria co-producing PulIA16C with PulK WT (-), PulKI9C, PulKL10C and PulKA11C. Cell extracts were analyzed using an anti-PulI (D) or anti-PulK antibody (E). (F) Pull down of cross-linked PulIL10C and PulJL16C using PulK-His₆ and Ni-magnetic beads. Theoretical molecular mass of PulI, 12.9 kDa; PulJ, 21.4 kDa; PulK, 35.2 kDa.

Figure 5 Molecular dynamics (MD) of full-length GspI, GspJ and GspK in a model membrane. (A) Initial state of the trimeric complex of GspI, GspJ and GspK after adding POPE lipids with arrows indicating the N-termini. (B-D) Snapshot of the final structure of GspI, GspJ or GspK from MD simulations of each protein alone. (E-F) Position of each

residue (mean + SD) along the bilayer normal in the starting structure and last 5 ns in GspI, GspJ and GspK alone simulations (E) or the GspI-GspJ-GspK complex simulation (F). Dashed lines show the lipid glycerol backbone atoms position. **(G-H)** Minimum distance between residues in the TM segments of GspI and itself, GspJ or GspK for the GspI-GspJ-GspK complex simulation (G) and two GspI-GspJ dimer simulations (H). **(I)** As in (G-H) but for residues targeted for cross-linking experiments. **(J)** Snapshot of GspI-GspJ-GspK MD simulation showing the membrane deformation induced by GspK.

Figure 6 Structure of the GspI-GspJ dimer and GspI-GspJ-GspK trimer in the membrane. **(A)** Representative structure of GspI-GspJ dimer in the main cluster of the two merged simulations (left). Representative structure of GspI-GspJ-GspK in the main cluster of the MD simulation (right). **(B)** Representative structures of the three main clusters of the GspI-GspJ and GspI-GspJ-GspK simulations (red) and the six main clusters of the GspJ alone simulation (gray), shown in the same view as (A). **(C)** Representative structures of GspI and GspK in the three main clusters of the GspI-GspJ and GspI-GspJ-GspK simulations (red) and the three main clusters of the GspI or GspK alone simulations (gray). **(D)** Snapshots of the final structures in two GspI-GspJ MD simulations.

Figure 7 Effect of Pull residue substitutions on pilus assembly. **(A)** ETEC's GspI-GspJ-GspK complex showing residues E35, W42, E45 and N46 in GspI. **(B)** Two-hybrid assay of T25-PulJ chimera and WT T18-PulI or indicated mutant variants. Zip-T18 and Zip-T25 are used as positive control and T25 as negative. **(C)** Cysteine cross-linking of PulJL16C with PulIL10C or its indicated double substituted variants. **(D)** Pilus assembly in strains co-producing PulJ or PulJ and PulK with WT Pull or its variants N46K and W42A/N46K. **(E)** PulG percentage in SF (mean + SD) from three independent experiments like the one in (E). **(F)** PulA immunodetection in cell extracts and supernatants (C, SN) of *E. coli* expressing all *pul* genes with a *pull* deletion complemented with WT *pull* or indicated mutant derivatives.

Figure 8 Pseudopilus assembly model. **(A)** Length control of pseudopilus based on the number of initiation sites. In WT bacteria the minor pseudopilin complex (labeled IJK) initiates assembly of H and G pseudopilins into pili of a certain length. **(B)** In the absence of the minor pseudopilins, spontaneous but sporadic initiation leads to assembly of long pili driven by cell-accumulated major pseudopilins. **(C)** During the first initiation step Pull (I) and PulJ (J) interact, leading to a 1-nm shift between their transmembrane segments. **(D)** PulK (K) is partially extracted from the membrane upon Pull-PulJ binding, forming a preassembled trimeric tip complex. **(E)** The upward movement of the minor pseudopilins upon PulK binding could activate the assembly platform by coordinating structurally the assembly of incoming pseudopilins and by bringing the assembly ATPase in contact with the membrane phospholipids, which is required for the activation of ATP hydrolysis.

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

Intact bacteria

Spheroplasts

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

A **B** **C** **D** **E**