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Minor pseudopilin self-assembly primes type II secretion pseudopilus elongation

David A Cisneros, Peter J Bond, Anthony P Pugsley, Manuel Campos and Olivera Francetic

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28 June 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the long delay in getting back to you with a decision: as I told you, we had some difficulty in finding three appropriate referees, and in addition, the final report was late in coming in. However, we do now have the comments from all three referees, which are enclosed below.

As you will see, all three reviewers express interest in your work, but all three also raise a number of serious concerns with the manuscript that would need to be addressed in a revised version before we could consider publication here. I would like to draw your attention particularly to the general comments of referee 2. Firstly, he/she questions why you have not used pullulanase secretion as an assay for T2SS functionality. As I understand it, all your experiments are conducted in *E. coli*, using over-expression of the *Klebsiella* T2SS operon. This is actually not sufficiently clear from the results section, where you simply refer to over-expression 'in bacteria', rather than stressing that this is a heterologous system. Moreover, if there is a way in which you are able to analyse T2SS functionality in a less permissive system, I would strongly encourage you to do this. Secondly, this referee (as well as the others) highlights a number of places where the data are perhaps over-interpreted; it would be important to ensure that the text is toned down where appropriate. Finally, referee 3 points out a number of important missing controls and quantifications.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on

our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The authors have extensively analyzed the contributions of minor pseudopilus subunits for assembly of the type II secretion pseudopilus of *Klebsiella oxytoca*. A series of detailed experiments are described, providing evidence for nucleation of filament assembly by the pseudopilus subunits PulI and PulJ at the inner membrane. PulK binding to this complex induces partial extraction of the I/J complex from the membrane and represents and initiating event for pseudopilus polymerization. Overall the experiments are well described and the findings will be of considerable interest for many researchers interested in morphogenesis of molecular machines and organelles. I am not an expert on molecular simulations and will defer to other reviewers to evaluate these data. The microscopy and biochemical studies strongly support the proposed functions of the minor pseudopilus subunits in the initiation step of assembly. I have only a few minor comments for the authors to address in the interest of improving this very nice study further.

1. Line numbers should be supplied to facilitate the review process.
2. Pg. 4. Bottom. "Mutant" refers to a strain, "mutation" to a gene, e.g., "pseudopilin deletion mutations" not 'mutants'.
3. Pg. 5. End of first paragraph. The data suggest that the pseudopilins are important (not even required absolutely) for pilus assembly, but these data do not provide any evidence for interactions among these subunits. Perhaps "functional interaction" ?
4. Expression of the minor pseudopilin genes from the lacZ promoter only weakly restores pilus assembly - any explanation? Regardless, it's important to note that piliation is not WT.
5. The HIJK mutant makes pili, albeit only a few. This is important to keep in mind, as throughout the manuscript it is stated that the minor pseudopilins are essential or required for pilus assembly - not true.
6. Was the PulG-BAP fully functional, e.g., did it display WT properties? More to the point, while certainly a nice way to visualize pili, the IFM studies seem to be as good. Was there a specific reason for including these data other than to highlight the use of this novel assay?
7. Pg. 6. The lack of detection of pilus on the surface of intact bacteria shown in the Suppl file should be moved to Fig. 3. Why separate these data - suggest incorporating the Suppl. File into Fig. 3 entirely.
8. Pg. 9. Middle. The correlations of crosslinking data and the structural findings are striking, although somewhat unbelievable with regard to the crosslinking findings. In a helical wheel depiction of the alpha helix, the adjacent residues are not near each other, so it's somewhat surprising that the crosslinking patterns of adjacent residues are so similar - this is suggestive of considerable flexibility in the helix-helix contacts. A stronger case that a face of the helix forms specific interhelical contacts would be made by tests of disulfide crosslinking of substituted cys residues all along the predicted helix=helix interface. Fundamentally, the disulfide crosslinking study is really too limited for the conclusions drawn, as is the bacterial two-hybrid study.
9. In the model, it isn't clear what role GspE plays. Is ATP hydrolysis required for extraction of the

pseudopilins from the membrane? If so, how do the authors envision this to occur? If not, what's the function of this essential ATPase?

Referee #2:

The manuscript by Francetic and colleagues addresses an important and poorly understood aspect of pilus/pseudopilus biogenesis, which is the initiation of filament assembly. Using a series of genetic and biochemical analyses specific interactions are identified between the minor pseudopilin subunits PulI, J and K from *Klebsiella oxytoca* and a model is presented for how this nucleation complex might initiate filament assembly in the Type II (T2S) secretion system. The protein:protein interaction results are convincing and contribute to our understanding of this system but I have reservations about some key interpretations in the paper.

1. Instead of using pullulanase secretion as an assay for the functional requirements of the minor pseudopilins, pseudopilus assembly was assessed. This requires overexpression of the major pseudopilin PulG (or of the minor pseudopilins), and is thus a very permissive system that allows some pilus assembly to proceed even in the absence of all of these proteins. Provide a rationale for why pullulanase secretion was not used to identify interactions and disrupting mutations.

2. The MD studies were performed using the published structure of a complex between the T2S minor pseudopilins GspI, J and K from ETEC (Korotkov and Hol, 2008), to which idealized α-helices were added as the N-terminal tails. MD simulations were performed with GspI and J, and with all three proteins in lipid bilayers. Minimal changes were observed with respect to the interactions among the N-terminal tails, yet throughout the text, the protein:protein interactions present in the final structure are referred to as having been identified from the MD studies, when in fact they were present all along. An example of this overinterpretation of the MD results is the section header "Molecular dynamics of minor pseudopilin complexes in the membrane reveals interactions between their transmembrane segments".

3. As in their 2010 PNAS paper, the authors introduce cysteines at strategic positions in the TM region and demonstrate that these cysteines form cross-links between the pseudopilins in a highly specific manner that is consistent with the subunit: subunit interactions identified in the crystal structure modeling (or, as the authors say, from the MD results). The authors claim in many places that the staggered nature of these interactions is induced upon binding of the two subunits. This is an attractive hypothesis but no proof is provided for this induced fit. The staggered nature of these interactions may simply be defined by the size and orientation of the globular domains with respect to the lipid bilayer, thereby causing their α-helical tails to lie at different levels in the membrane. Indeed, in the GspIJK structure, in which the N-terminus of GspK is at the lowest level, followed by GspI then GspK, the bottoms of globular domains of I and J appear at the same level suggesting that their orientation in the lipid bilayer is similar to their orientation in the crystal structure. This structure suggests that the tails of I and J are staggered prior to their interaction. This may not be the case for K, which does appear to be "extracted" from the bilayer in the crystal structure.

Additional comments/questions/suggestions are as follows, listed as they arise in the text.

P. 5. "this showed that the minor pilins are required for pilus initiation." If they are required why are there pili present? Perhaps say "required for efficient initiation".

P. 6. "PulI and PulJ together promoted efficient PulG assembly" - here and elsewhere, change to "PulG pilus assembly".

P. 7. That a kink arises at G36 of GspJ is not surprising. Does this kink appear in the absence of the other subunits? If so, it is not induced upon interaction with these subunits or even with the lipid bilayer, but is likely due to the helix-disrupting propensity of glycine. Where is the "slight kink" in GspI (i.e. at which residue)?

P. 9. "The same pattern of cross-linking of all PulI cysteine-substituted variants was observed in the presence of the Pul secretion (Supplementary Fig. 5)." Provide some details on the experiment here.

P. 10. An E5K substitution was introduced in PulI in an attempt to disrupt its binding to PulJ, but no figure is provided for this residue showing that it may be involved in subunit:subunit interactions. Such a figure is provided in Korotkov and Hol (2008), showing the E5 of Gsp J is close to F1 of I, and E5 of I is close to M1 of K, so presumably this was observed for the current study in the model used in the MD simulation. Were these interactions maintained in the final MD complex? The N-termini appear splayed at the end of the simulation. Is this the case? If so, this may be due to the fact that idealized α-helices were used. It appears that the MD simulation introduces some curvature to the helices. Is this what is meant by the kinks?

P. 10. The ability of the PulI-E5K variant to interact with PulJ and to complement the ΔpulI strain for pilus assembly may be an artifact of the over-expression system. It would be very interesting to know if the PulI-E5K variant could function in secretion.

P. 11. "In conclusion the requirement of the PulI, PulJ and PulK for efficient pilus inititation correlates with their absolute requirement for protein secretion." Provide a reference for their "absolute requirement for pilus secretion" as secretion was not tested here.

P. 13. "All T4P 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." Note that this set of genes is not present in Type IVb pilus operons, just Type IVa.

The methods section is very sparse. Provide details for overexpression of PulG in cells shown in Figs. 1 and 2 and indicate in the results when PulG is overexpressed. Provide more detailed methods for the 2-hybrid system. How were the plasmids generated? Provide the primer sequences.

Figure 2 should include the "wild type" K12 strain expressing the complete pul operon. Does the ΔpulHIJK+pulHIJK strain look like wild type?

Fig. 4. The text is very small and hard to read. Color the carbons in the side chains the same color as in the backbone ribbons so that its clear which side chain is associated with which helix. Remove the hydrogens.

Fig. 6. Does the (-) sign indicate that no cysteines have been introduced or that no PulI is present because the plasmid isn't present? The former should be the case.

Supplementary Fig. 6. Indicate hetero and homodimers.

Supplementary movies. The phospholipid head groups of the bilayer are shown in Movie 1 but not in Movie 2. Does the black region in Movie 2 represent the acyl phase or the entire bilayer. If its the entire bilayer, the N-terminal tails appear much more exposed in the periplasm in Movie 2 compared to Movie 1.

Referee #3:

In this study Cisneros et al have investigated the role of the minor pseudopilins PulI, PulJ and PulK in the assembly and function of the major pseudopilin PulG of the type II secretion system (T2SS). The manuscript is well written and the experiments are logical. The findings are of potential interest; however, some of the results are confirmatory and some of the experiments could be more informative if additional controls were included and the data were quantified.

Comments

Page 4, third paragraph: Modify the sentence that ends with 'we studied their function in the T2SS of *K. oxytoca*'. The studies were performed with *E. coli* expressing *K. oxytoca* genes.

Figure 1: Two controls are lacking. For both the shearing experiment and the fluorescence microscopy, include a ΔpulG mutant as a control for the antibody specificity and a strain that expresses only pulG to demonstrate that removal of all T2S genes results in complete loss of surface filaments. The PulG-specific bands on the blot in panel A should be quantified and the average from

at least three different experiments should be presented. The results could be presented as 'sheared pili as a fraction of total pili'. This will account for potential differences in expression levels between different strains tested and will allow for direct comparison of different strains even when they are analyzed on different blots. Why are the pili longer for the mutants? Do the wild type filaments break more easily? Why are the cells of ΔpulI and ΔpulK longer than wild type cells? Is there a cell division defect?

Figure 2: Again, the data should be quantified. The experiments presented in panels A and B should be repeated at least three times and the PulG-specific bands should be quantified and the average presented. When comparing with the results presented in figure 1 panel A it appears as if ΔpulHIJK is not fully complemented with the plasmid carrying the pulHIJK genes. Also, there appear to be many fewer filaments for the complemented ΔpulHIJK strain compared to the wild type strain. The results from the immunofluorescence microscopy would be more informative if the filaments are counted and presented as 'number of filaments per cell'. Is the secretion of pullulanase fully complemented with the plasmid carrying the pulHIJK genes?

Figure 3: please provide information as to how the spheroplasts were isolated. What type of quality control was performed to determine the efficiency of outer membrane disruption? Please include controls:1) a strain that does not have the pulG gene to show specificity of the assay and 2) a strain that is expressing pulG only and should not produce any periplasmic filaments.

Figure 6 and page 9: Provide information on the predicted sizes for PulI, PulJ and PulK and the expected sizes for cross-linked products. In panel B, why is PulJL16C-His6 not detected when produced in the absence of PulI?

Figure 7: Quantify the data in panel D.

Figure 8: Can the authors experimentally demonstrate the existence of multiple initiation sites in the membrane with the use of PulI antibodies and immunofluorescence microscopy?

Supplementary figure 1: An alternative procedure for labeling and detection ofsurface-exposed PulG is presented. Although very promising, a few negative controls are needed for the fluorescence microscopy. A strain not expressing PulG-BAP would provide specificity for the assay. Another control to account for the specificity of the labeling would be using ΔpulHIJK::pulG-+ pulHIJK, but omitting the addition of purified biotin ligase.

1st Revision - authors' response 14 October 2011

Answers to referees
Manuscript EMBOJ-2011-78128R

Referee #1:

The authors have extensively analyzed the contributions of minor pseudopilus subunits for assembly of the type II secretion pseudopilus of Klebsiella oxytoca. A series of detailed experiments are described, providing evidence for nucleation of filament assembly by the pseudopilus subunits PulI and PulJ at the inner membrane. PulK binding to this complex induces partial extraction of the I/J complex from the membrane and represents and initiating event for pseudopilus polymerization. Overall the experiments are well described and the findings will be of considerable interest for many researchers interested in morphogenesis of molecular machines and organelles. I am not an expert on molecular simulations and will defer to other reviewers to evaluate these data. The microscopy and biochemical studies strongly support the proposed functions of the minor pseudopilus subunits in the initiation step of assembly. I have only a few minor comments for the authors to address in the interest of improving this very nice study further.

1. *Line numbers should be supplied to facilitate the review process.*
The line numbers have been introduced as suggested.

2. Pg. 4. Bottom. "Mutant" refers to a strain, "mutation" to a gene, e.g., "pseudopilin deletion

mutations" not 'mutants'.
Such errors have been corrected throughout the manuscript.

3. Pg. 5. End of first paragraph. The data suggest that the pseudopilins are important (not even required absolutely) for pilus assembly, but these data do not provide any evidence for interactions among these subunits. Perhaps "functional interaction ?

The phrase has been corrected according to the reviewer's suggestion to: "All these results suggest a functional interaction between PulI, PulJ and PulK, which is required for efficient pilus assembly" (line 117).

4. *Expression of the minor pseudopilin genes from the lacZ promoter only weakly restores pilus assembly - any explanation? Regardless, it's important to note that piliation is not WT.*

This was a concern raised by that all three reviewers. After repeating these experiments and comparing pilus assembly in the minor pseudopilin mutant with WT, as suggested by the reviewers, we realized that this mutant produces less PulG for reasons that we do not fully understand, as sequence analysis of *pulG* gene and the upstream regions did not reveal any mutations. The comparison of the PulG levels in these strains is shown in the new Figure 2C. We also discuss this point in the manuscript (lines 134-137). This reduced production of PulG led to an apparent decrease in the number of surface pili in the complemented strain, which is more visible in the fluorescence microscopy images. However, owing to the careful quantification suggested by Reviewer #3, we found that pilus assembly efficiency expressed as the percentage of PulG in the sheared fraction does not differ significantly between WT and the complemented strain (new Fig 2D). Moreover, this mutant is complemented fully for pullulanase secretion (see below page 10 of this letter). Most importantly, comparison of the *ΔpulHIJK* strain complementation by the *pulIJ* and *pulHIJK* genes (which we think is the appropriate control here) supports our main conclusion that PulI and PulJ together promote pilus assembly.

5. The HIJK mutant makes pili, albeit only a few. This is important to keep in mind, as throughout the manuscript it is stated that the minor pseudopilins are essential or required for pilus assembly - not true.

This is an excellent point raised by Reviewer 1. Indeed, the occasional pili are assembled in the absence of minor pilins, suggesting they play a catalytic role in a process that involves PulG and other factors, presumably components of the assembly machinery. What we clearly see is that the absence of the minor pseudopilins affects dramatically the number of assembled pili. In the new version, the assertion that the minor pseudopilins are "required" or "essential" for pilus assembly was replaced by "required for efficient pilus assembly" or "efficient pilus initiation" (*e.g.* lines 117-118).

6. Was the PulG-BAP fully functional, e.g., did it display WT properties? More to the point, while certainly a nice way to visualize pili, the IFM studies seem to be as good. Was there a specific reason for including these data other than to highlight the use of this novel assay?

Although PulG-BAP was fully functional, the reviewer made a very pertinent remark that these experiments do not bring any new information. Therefore we decided to delete this supplementary figure and replace it with the original Fig. 1, which demonstrates the effect of overproduction of PulG relative to the other T2SS components. This is also an answer to the remarks of Reviewer 3, who requested more controls for the PulG-BAP experiment.

7. Pg. 6. The lack of detection of pilus on the surface of intact bacteria shown in the Suppl file should be moved to Fig. 3. Why separate these data - suggest incorporating the Suppl. File into Fig. 3 entirely.

These supplementary data have been included in the new Figure 3, as suggested by the Reviewer.

8. Pg. 9. Middle. The correlations of crosslinking data and the structural findings are striking, although somewhat unbelievable with regard to the crosslinking findings. In a helical wheel depiction of the alpha helix, the adjacent residues are not near each other, so it's somewhat surprising that the crosslinking patterns of adjacent residues are so similar - this is suggestive of considerable flexibility in the helix-helix contacts.

We agree with Reviewer 1 that the cross-linking study suggests considerable flexibility, which we believe to be a hallmark of hydrophobic interactions in the membrane environment. The

neighboring residues are localized on the same face of the alpha helix and the Cysteine substitutions may allow cross-linking due to the somewhat longer side-chains compared to the native residues. Using the same cysteine cross-linking approach, we also have evidence from interactions observed in the structure of the PulG pilus (Campos, *et al.*, 2010, PNAS, 107:13081) that the transmembrane segments make flexible contacts between neighbouring protomers. Double cysteine substitutions in positions 11 and 16 of PulG allowed the cross-linking of assembled pili, although less efficiently that those in positions 10 and 16. This occurred in the fully assembled pilus, where structural constraints might be higher compared to the membrane environment. Our cross-linking results are also in agreement with the molecular dynamics analysis, where GspI and GspJ explore several conformations in the dimer simulations.

However, to address this question, we performed oxidation experiments at reduced stringency, achieved by reducing the time of Cu-Phenanthroline treatment. These results, (included as the Supplementary Fig. 5) show that PulIL10C cross-links to PulJ L16C more efficiently than PulI A11C (lines 210-213). Therefore, as in the PulG pilus, there is a preference for the interaction between positions 10 and 16 in the GspI-GspJ dimer.

A stronger case that a face of the helix forms specific interhelical contacts would be made by tests of disulfide crosslinking of substituted cys residues all along the predicted helix-helix interface. Fundamentally, the disulfide crosslinking study is really too limited for the conclusions drawn, as is the bacterial two-hybrid study.

Although it would have been excellent to perform these experiments, and fully explore the relationship between the MD model and biological data, we would like to emphasize that our principal aim here was to show the staggered position of the PulI and PulJ TM segments. Indeed, we think that the cysteine cross-linking, although limited to one helical turn of PulI demonstrates this point unambiguously. Taken together, all the evidence, including the crystal structures of PulI and PulJ homologues in complex, the MD simulations, the position-specific cross-linking and the correlation of the cross-linked positions to the major pseudopilin pilus structure, converge to the conclusion of a staggered PulI-PulJ conformation. We were surprised to find these interactions also during the MD experiments, as probably the complex does not reach an equilibrium state at the end of the simulation. Nevertheless, our hope is that the readers would appreciate the potential of the MD simulations as tools to study structure-function relationship of membrane proteins linked to conformational changes that can only be studied *in silico*. Extensive validation of these models is in progress and more simulations are being performed to increase the statistical significance of the conformations probed by the membrane embedded pseudopilins. However, we feel that these studies are beyond the scope of the present article.

The bacterial two-hybrid analysis was used as an alternative means to study the interactions betweens pseudopilins that have been tested by cross-linking. Surprisingly, the interaction results also match the *in vivo* data shown in Fig. 2. We also show that PulI and PulJ interact in the membrane in the absence of the secretion system and the assembly ATPase. We are currently performing a more general two-hybrid study to analyze the interactions between all pairs of pseudopilins and also with the assembly platform components, which will be described in a future publication.

9. In the model, it isn't clear what role GspE plays. Is ATP hydrolysis required for extraction of the pseudopilins from the membrane? If so, how do the authors envision this to occur? If not, what's the function of this essential ATPase?

We show in the manuscript that the interactions between PulI and PulJ, and PulI and PulK do not require the secretion ATPase PulE or any other type II secretion proteins and as mentioned above, there is converging evidence that their TM segments are shifted when they bind. Very likely, the function of PulE is to extract the major (pseudo)pilins from the membrane to allow them to assemble into a filament. We envision a model where the binding of the minor pseudopilins at the level of the inner membrane occurs spontaneously without the addition of any external energy (and several experiments in our manuscript point to this) leading to the spontaneous partial extraction of PulK from the membrane to acquire a pseudopilus like structure. How this coordinates events downstream the formation of this complex at the level of the inner membrane assembly machinery is a very interesting question that we are currently exploring.

Furthermore, to address this question, we introduced the following sentence in the Introduction section: “Successive conformational changes of the ATPase presumably power the membrane assembly platform components to catalyze the addition of pilin monomers to elongate the fiber” (Line XX). We also introduced this sentence in discussion: “Interestingly, the T4P retraction

ATPase PilT (Campos *et al*, 2010; Craig *et al*, 2006; 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 (lines 439-442). More details of our model are also provided in lines 427 to 442.

Referee #2:

The manuscript by Francetic and colleagues addresses an important and poorly understood aspect of pilus/pseudopilus biogenesis, which is the initiation of filament assembly. Using a series of genetic and biochemical analyses specific interactions are identified between the minor pseudopilin subunits PulI, J and K from Klebsiella oxytoca and a model is presented for how this nucleation complex might initiate filament assembly in the Type II (T2S) secretion system. The protein:protein interaction results are convincing and contribute to our understanding of this system but I have reservations about some key interpretations in the paper.

1. Instead of using pullulanase secretion as an assay for the functional requirements of the minor pseudopilins, pseudopilus assembly was assessed. This requires overexpression of the major pseudopilin PulG (or of the minor pseudopilins), and is thus a very permissive system that allows some pilus assembly to proceed even in the absence of all of these proteins. Provide a rationale for why pullulanase secretion was not used to identify interactions and disrupting mutations.

It has been impossible so far to study the assembly of the T2SS major pseudopilin into periplasmic filaments. Therefore the only way to analyze pilus assembly by the T2SS is by overproducing it, which allows us to observe, purify and measure fibers extending beyond the cell surface. For this purpose, the DNA fragment with the complete set of *pul* genes under control of their native promoters was cloned on a moderate copy-number plasmid (pBR322) to preserve the regulation and the stoechiometry of the system. New tools will have to be designed to study the periplasmic fiber and we are working on them. Furthermore, by using diverse biochemical strategies, deletions and point mutations, we intended indeed to isolate this phenomenon from events downstream to pseudopilus elongation.

In the first version of the paper, we cited previous studies from the Pugsley lab showing that pilus assembly and protein secretion are defective in the *pulI* mutant and the protein secretion is null in the *pulJ* and *pulK* mutants. In the new version we analyze these mutants and in the same over-expression context, this time using the soluble (non-acylated) variant of PulA, which allows us to study secretion using a simple fractionation experiment. The secretion defects and the pilus assembly defects correlate very nicely. In particular the *pulH* mutant is very similar to WT in this context and the *pulI*, *pulJ* and *pulK* mutants are defective for both efficient pilus assembly and protein secretion (new Fig. 1). In addition, we show that in the less permissive, indeed physiological conditions, where the gene copy number is lowered to the chromosomal expression level in the *pcnB* mutant *E. coli*, the secretion defect of the *pulH* mutant is also more dramatic (Supplementary figure S1B-C). Presumably, this defect corresponds to a reduction of the protein secretion rate.

Most importantly, as suggested by reviewers, we included data on pullulanase secretion in the *pulI* point mutants, which is unaffected, even under the most stringent conditions (Fig. 7F). This point is now discussed in the last paragraphs of the Results (lines 327-334). We show in the new Figure 7 that this is probably due to the fact that PulK rescues the efficient pilus assembly. This further suggests that the functional interaction *in vivo* between PulI, PulJ and PulK to promote efficient pilus assembly is necessary for pullulanase secretion.

2. The MD studies were performed using the published structure of a complex between the T2S minor pseudopilins GspI, J and K from ETEC (Korotkov and Hol, 2008), to which idealized alpha-helices were added as the N-terminal tails. MD simulations were performed with GspI and J, and with all three proteins in lipid bilayers. Minimal changes were observed with respect to the interactions among the N-terminal tails, yet throughout the text, the protein:protein interactions present in the final structure are referred to as having been identified from the MD studies, when in fact they were present all along. An example of this overinterpretation of the MD results is the section header "Molecular dynamics of minor pseudopilin complexes in the membrane reveals interactions between their transmembrane segments".

Indeed, Korotkov and Hol have already modelled the TM segments onto the globular domains in the crystal structure and showed that the proteins are shifted to form the pilus-like complex. However, no details were provided about the specific residue contacts in the TM segments

which were the focus in our study of the full-length membrane embedded proteins. These are the interactions that we wanted to predict based on the MD simulations, in order to be able to test them in our assays. Nevertheless, to address this criticism, we placed the paragraph describing the MD simulations after the functional, interaction and cross-linking analysis. Furthermore, we re-wrote this section (line 246-294) starting from the header, which is now called "*Molecular dynamics of full-length minor pseudopilin complexes*". New MD simulations to support our self-assembly model (see below) are also included but we present them as *in silico* predictions, which will require validation *in vivo* (line 290-294). We would also like to point out that in this study we only placed these proteins in the modelled membrane to study their behaviour, the degree of their interaction and insertion in the bilayer. We reasoned that initially all these proteins must have been fully inserted in the membrane in the same fashion, due to their positively charged N-terminal peptide. We no longer make the claim that the MD study was used to predict specific contacts between the TM segments. Instead of snapshots of GspI and GspJ TM segments, contact maps of the final models were also added (new Fig 5) but mainly as a tool to observe the TM segments shift (see below).

3. As in their 2010 PNAS paper, the authors introduce cysteines at strategic positions in the TM region and demonstrate that these cysteines form cross-links between the pseudopilins in a highly specific manner that is consistent with the subunit subunit interactions identified in the crystal structure modelling (or, as the authors say, from the MD results). The authors claim in many places that the staggered nature of these interactions is induced upon binding of the two subunits. This is an attractive hypothesis but no proof is provided for this induced fit. The staggered nature of these interactions may simply be defined by the size and orientation of the globular domains with respect to the lipid bilayer, thereby causing their alpha-helical tails to lie at different levels in the membrane. Indeed, in the GspIJK structure, in which the N-terminus of GspK is at the lowest level, followed by GspI then GspK, the bottoms of globular domains of I and J appear at the same level suggesting that their orientation in the lipid bilayer is similar to their orientation in the crystal structure. This structure suggests that the tails of I and J are staggered prior to their interaction. This may not be the case for K, which does appear to be "extracted" from the bilayer in the crystal structure.

Several arguments lead us to think the TM segments of the three pseudopilins might be inserted at similar levels in the membrane before they interact. First, during biogenesis, these proteins are inserted in the IM as precursors with an N-terminal positively charged signal anchor, which presumably interacts with the negatively charged phospholipid interface. Second, the prepilin peptidase cleavage site should also be localized at this interface. Third, the extent of insertion of a TM segment in the membrane plane is very likely determined by the hydrophobic nature of its residues. It is the case that the TM segments of the Pul pseudopilins are highly similar, with the exception of PulK, which doesn't have the conserved E5 residue (this could favour the conformational change required to partially extract it from the membrane upon binding).

Reviewer 2 suggests that it can be inferred from the GspI-J-K structure that GspI and GspJ are staggered prior to their interaction in the membrane. To address this criticism, we performed MD simulations of the three pseudopilins alone, starting from their conformation in complex, and allowing them to re-equilibrate in the membrane in the absence of their partners (lines 247-258). These simulations showed that these proteins "reintegrate" to the same level in the membrane plane from the starting model independent of the size and orientation of their globular domains in both soluble or full-length models (new Fig. 5). The difference of the membrane insertion levels between the monomers and the trimers is illustrated in Fig. 5E. Fig. 5F shows that indeed the GspI and GspJ do not seem extracted from the membrane, but rather appear to accommodate by modifying their position within the membrane. However, GspK is partially extracted, as rightfully pointed out by Reviewer 2.

Furthermore, by cluster analysis we identified conformational changes in the structure of GspJ and to a lesser extent GspI, which occupy different conformations when they are simulated alone or in complex with one or two of their partners. The TM segment of GspJ remained straight in the monomer simulation and was bent in the oligomer simulations. This is of course no proof of induced fit, but it does suggest that the interaction of PulI and PulJ mediated by the globular domains has structural consequences at the level of the TM segments. This is shown now in the new Figure 6 of the Results. GspI appears to interact with the phospholipid head-groups via the β3-β4 loop, but the globular domain of GspJ makes no such contacts. Interestingly, the GspI-GspJ simulations show that the complex tilts in the membrane and that these interactions may be responsible for the tilting, which is yet another conformational change, not visible in the monomer simulations. This presumably allows the GspI-GspJ complex to maximize their contact with the

lipid bilayer. In the new Figure 5, we also show how the interactions of the TM segments remain shifted, by looking how far from the diagonal they appear in the interaction maps. We hope that together these results support our model of a conformational change upon GspI and GspJ binding.

Additional comments/questions/suggestions are as follows, listed as they arise in the text.

P. 5. "this showed that the minor pilins are required for pilus initiation." If they are required why are there pili present? Perhaps say "required for efficient initiation".

The phrase was rewritten as follows: " This further shows that minor pseudopilins are required for efficient pilus initiation, but not for its elongation" in line 115. Thereafter, we refer to complementation by all minor pseudopilin genes as being able to affect/restore "efficient pilus assembly".

P. 6. "PulI and PulJ together promoted efficient PulG assembly" - here and elsewhere, change to "PulG pilus assembly"

The expression has been corrected throughout as suggested.

P. 7. That a kink arises at G36 of GspJ is not surprising. Does this kink appear in the absence of the other subunits? If so, it is not induced upon interaction with these subunits or even with the lipid bilayer, but is likely due to the helix-disrupting propensity of glycine.

The kink in GspJ does not appear when GspI or GspK are not present, as shown in the new Fig. 6B, suggesting that the constraint is induced by the presence of the other two pseudopilins. This and other conformational changes are now discussed in the paragraph beginning at line 276.

Where is the "slight kink" in GspI (i.e. at which residue?)?

The reviewer is raising an important point. We were referring to the fact that the TM segment of GspI does not remain straight after MD simulation. However, as the new MD simulations show, this partially occurs in both GspI alone or GspI with GspJ and GspI-GspJ-GspK (although the second main cluster of the GspI alone simulation appears quite straight). This suggests that this is a conformation which is intrinsic to GspI. We address this point now in the above-mentioned paragraph (line 281). We also introduced the term "curvature" instead of "kink" as suggested by the reviewer after looking at the structures derived from the clustering analysis. The curvature of GspI is situated at different parts of the N-terminal α-helix depending of the cluster analyzed. In some of the clusters it is centered around Gly26, but not in all of them.

P. 9. "The same pattern of cross-linking of all PulI cysteine-substituted variants was observed in the presence of the Pul secreton (Supplementary Fig. 5)." Provide some details on the experiment here.

Details added in lines 208 to 210.

P. 10. An E5K substitution was introduced in PulI in an attempt to disrupt its binding to PulJ, but no figure is provided for this residue showing that it may be involved in subunit:subunit interactions. Such a figure is provided in Korotkov and Hol (2008), showing the E5 of Gsp J is close to F1 of I, and E5 of I is close to M1 of K, so presumably this was observed for the current study in the model used in the MD simulation. Were these interactions maintained in the final MD complex?

Our initial model was generated with absolutely no restrains, including lack of interaction between GspI or GspJ E5 and the adjacent N-terminus. As far as we understood, Korotkov and Hol (2008) used idealized helices to model the straight helices; nothing is said about any kind of refinement of this structure or rotational conformers of side chains that match this interaction. In our membrane-embedded model, both GspI and GspJ E5 are "neutralized" by contacting the phospholipid headgroups. In addition, they do not appear close in the contact maps shown in Fig. 5G or H. In the PulG pilus (Campos *et al.*, 2010, PNAS) and in a PilA membrane-embedded monomer model, which we refer to in the new version (Lemkul and Bevan, 2011), its shown that E5 can also be neutralized intramolecularly by the N-terminal positive charge.

The N-termini appear splayed at the end of the simulation. Is this the case? If so, this may be due to the fact that idealized alpha helices were used. It appears that the MD simulation introduces some curvature to the helices. Is this what is meant by the kinks?

The N-termini do appear a bit splayed in the initial model but thanks to re-arrangement (and induced curvature) of TM segments during MD simulations, their final conformation is more compact (compare new figure 5A with new figure 6A-B). As discussed above the curving of GspJ is

induced in the MD simulations upon interaction with GspI and GspK.

P. 10. The ability of the PulI-E5K variant to interact with PulJ and to complement the ΔpulI strain for pilus assembly may be an artefact of the over-expression system. It would be very interesting to know if the PulI-E5K variant could function in secretion.

We show here that the E5K mutation has no incidence on the PulA secretion even under the most stringent conditions and it does not affect the interaction between PulI and PulJ, which is presumably dependent on the globular domains of these proteins. This is strikingly different from the E5A mutation of PulG, which abolished pilus assembly, an effect that cannot be explained only by structural constraints in the assembled pili as discussed previously (Campos *et al*., 2010).

P. 11. "In conclusion the requirement of the PulI, PulJ and PulK for efficient pilus inititation correlates with their absolute requirement for protein secretion." Provide a reference for their "absolute requirement for pilus secretion" as secretion was not tested here.

In this version of the manuscript PulA secretion was assayed, as explained above.

P. 13. "All T4P 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." Note that this set of genes is not present in Type IVb pilus operons, just Type IVa.

The sentence was rewritten as suggested by the reviewer as follows (line 443): "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). "
Nevertheless, we should point out that some type IVb systems have additional pilins that may play and analogous role.

The methods section is very sparse. Provide details for overexpression of PulG in cells shown in Figs. 1 and 2 and indicate in the results when PulG is overexpressed. Provide more detailed methods for the 2-hybrid system. How were the plasmids generated? Provide the primer sequences.

The Methods section was provided with more details. In the new Figures 1 and 2 PulG was produced at the same ratio relative to other T2SS components. Providing *pulG* on plasmid pSU18 under p_{lacZ} promoter control results in PulG overproduction relative to the *pul* genes and is only used in Supplementary figures S1 and S2. Overproduction of PulG is also specified in the new version (lines 111-114 and 141-143). Details of the bacterial two hybrid assay were provided. The primer list for the two-hybrid constructs has been added in the Supplementary Table 2.

Figure 2 should include the "wild type" K12 strain expressing the complete pul operon. Does the ΔpulHIJK+pulHIJK strain look like wild type?

A detailed answer to this point is provided in the answer to point 4 of reviewer #1 (page 2 of this letter).

Fig. 4. The text is very small and hard to read. Color the carbons in the side chains the same color as in the backbone ribbons so that its clear which side chain is associated with which helix. Remove the hydrogens.

This figure has been replaced by the interaction maps in the new Fig. 5, which give a more global view of the interactions between residues. A zoom is shown on the residue interactions within the TM segment, which were tested by cross-linking.

Fig. 6. Does the (-) sign indicate that no cysteines have been introduced or that no PulI is present because the plasmid isn't present? The former should be the case.

In Fig. 6C the (-) sign indicated the empty vector. We included this control because there is an unspecific band reacting with the antibody that migrates similar to PulI monomer. It was also included in Fig. 6B for congruency. Correct labels were introduced in the new Fig. 4. In Fig. 6D-E the (-) sign indeed indicated the WT PulK, as suggested by the reviewer. A parenthesis to indicate this was introduced in the new figure legend.

Supplementary Fig. 6. Indicate hetero and homodimers.

This has been corrected in the new Supplementary figure S3

Supplementary movies. The phospholipid head groups of the bilayer are shown in Movie 1 but not in

Movie 2. Does the black region in Movie 2 represent the acyl phase or the entire bilayer. If its the entire bilayer, the N-terminal tails appear much more exposed in the periplasm in Movie 2 compared to Movie 1.

In the trimer movie (now movie S2), only the water molecules are shown, which extend to the phospholipid headgroups but not to the acyl phase. Therefore the black region does denote the hydrophobic phase, as pointed out by the reviewer. In both dimer and trimer simulations the proteins are initially embedded in the membrane to the same level.

Referee #3:

In this study Cisneros et al have investigated the role of the minor pseudopilins PulI, PulJ and PulK in the assembly and function of the major pseudopilin PulG of the type II secretion system (T2SS). The manuscript is well written and the experiments are logical. The findings are of potential interest; however, some of the results are confirmatory and some of the experiments could be more informative if additional controls were included and the data were quantified.

Comments

Page 4, third paragraph: Modify the sentence that ends with 'we studied their function in the T2SS of K. oxytoca'. The studies were performed with E. coli expressing K. oxytoca genes.

The sentence was changed to "To understand the molecular role of minor pseudopilins, we studied their function in the T2SS of *K. oxytoca,* functionally reconstituted in *E. coli*." (lines 82-84).

Figure 1: Two controls are lacking. For both the shearing experiment and the fluorescence microscopy, include a ΔpulG mutant as a control for the antibody specificity and a strain that expresses only pulG to demonstrate that removal of all T2S genes results in complete loss of surface filaments.

We repeated these experiments in conditions of *pul* gene expression under control of an endogenous promoter in pBR322. The results, presented in the new Fig. 1 now have the controls requested. The Fig. 1 in the previous version of the paper is presented as the new Supplementary Fig. 1. Comparing the two figures provides evidence for an effect of PulG levels on the length of pili.

The PulG-specific bands on the blot in panel A should be quantified and the average from at least three different experiments should be presented.

The quantification has been performed on all main text data and the number of experiments as a basis for the quantification is indicated in the corresponding Figure legends as requested by the Reviewer. In Fig. 1D it must be noted that we are able to see differences in pilus assembly by western blot, when in the old version of the manuscript we could not. This is due to the fact that we reduced the amount of material loaded in the SDS-PAGE gels, which is also indicated in the new figure legends.

The results could be presented as 'sheared pili as a fraction of total pili'. This will account for potential differences in expression levels between different strains tested and will allow for direct comparison of different strains even when they are analyzed on different blots.

As suggested by the reviewer, we expressed the results as total PulG versus PulG in the sheared fraction. (We believe that the cell-associated PulG is largely unassembled into pili.) Indeed, we do find that this representation allowed a better comparison between different strains and conditions (as exemplified by experiments shown in Fig. 1-2 and supplementary Fig. S1-S2).

Why are the pili longer for the mutants? Do the wild type filaments break more easily?

Our model predicts that the longer pili arise from the lack of initiation sites due to the accumulation of PulG, as explained in Fig. 8. This is more obvious when PulG is overproduced (compare the mutants in the new Figure 1 with new Supplementary Fig. S1). We have no reason to think that PulG filaments should break more in the WT that in the mutants. This would imply that the minor pilins cause this fragility presumably by inserting in the middle of the filament (rather than the top). However, no minor pseudopilin have ever been found in extaracellular T2SS pili. Nevertheless, we cannot totally exclude this possibility, although it would be difficult to demonstrate.

Why are the cells of ΔpulI and ΔpulK longer than wild type cells? Is there a cell division defect?

E. coli bacteria overproducing the T2SS have a broader distribution of sizes than wild type *E. coli*. This is more dramatic when bacteria are selected on chloramphenicol, as shown in the old Figure 1. As shown in the new Figures 1 and 2 all bacteria have similar sizes (which are selected with ampicillin).

Figure 2: Again, the data should be quantified. The experiments presented in panels A and B should be repeated at least three times and the PulG-specific bands should be quantified and the average presented.

This has been done and is presented in the new Figure 2.

When comparing with the results presented in figure 1 panel A it appears as if ΔpulHIJK is not fully complemented with the plasmid carrying the pulHIJK genes. Also, there appear to be many fewer filaments for the complemented ΔpulHIJK strain compared to the wild type strain.

Please see answer to point 4 of reviewer #1 (page 2 of this letter).

The results from the immunofluorescence microscopy would be more informative if the filaments are counted and presented as 'number of filaments per cell'.

This is a very good suggestion, which we followed, and modified Figures 1 and 2 accordingly. It does show where the defect in the tip pseudopilin mutants lies, as there is a direct correlation between the number of pili and initiation of assembly.

Is the secretion of pullulanase fully complemented with the plasmid carrying the pulHIJK genes?

Yes, it is, as shown in this figure of the immunodetection of PulA in supernatants of this mutant complemented with *pulHIJK* in a plasmid. This experiment has been done in "high copy", to mimic the conditions of the pilus assembly experiment.

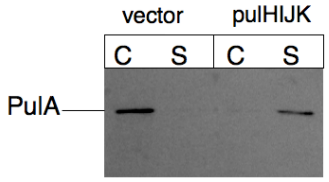

Figure 3: please provide information as to how the sphaeroplasts were isolated. What type of quality control was performed to determine the efficiency of outer membrane disruption?
Please include controls:1) a strain that does not have the pulG gene to show specificity of the assay and 2) a strain that is expressing pulG only and should not produce any periplasmic filaments.

The method used to make sphaeroplasts is described in a new paragraph of Materials and methods (lines 528-536). The efficiency of outer membrane disruption was monitored by light microscopy as described by Birdsell and Cota-Robles (1967). In Fig. 3, all bacteria are present in the form of sphaeroplasts as shown by their round shape and PulG of their surface. As Fig.3 shows the analysis of periplasmic fibers, we did not add an extra negative control in the same experiment. However, the same anti-PulG antibodies were used throughout the study and their specificity is now shown in Fig. 1, as rightfully requested by the Reviewer 3. In our opinion, the most important control in Fig. 3 is that periplasmic pilus assembly is restored when the *ΔpulD/ΔpulHIJK* mutant is complemented in trans with *pulHIJK*. The striking comparison with the intact cells, now provided in the new Fig. 3 as suggested by Reviewer 1 illustrates the efficiency of the sphaeroplasting very nicely.

Figure 6 and page 9: Provide information on the predicted sizes for PulI, PulJ and PulK and the expected sizes for cross-linked products.

The expected sizes of pilins and heterodimers in the cross-linking experiments are now provided in the new version (Fig. 4 and lines 200 and 228).

In panel B, why is PulJL16C-His6 not detected when produced in the absence of PulI?

Lane 1 was mislabeled by mistake because it contains the empty vector control (Not PulI or PulJ), we included this control because we had included it in the immunoblot in the now new Fig 4C. Please refer to answer to comment entitled "Figure 6" of reviewer #2 to see why we included

this control (page 8 of this letter).

Figure 7: Quantify the data in panel D.

Data has been quantified in the new Figure 7 and new Supplementary Fig. S5

Figure 8: Can the authors experimentally demonstrate the existence of multiple initiation sites in the membrane with the use of PulI antibodies and immunofluorescence microscopy?

We think that the proposed experiment represents technical challenges. It will be difficult to distinguish at the level of the inner membrane unassembled and assembled PulI, PulJ and PulK. We have concerns that epifluorescence would not have the resolution high enough to observe this binding event. This complex is presumably smaller than 6 nm, which is probably out of reach for even the most advanced microscopy techniques.

Previously we used GFP fusions to visualize the components of the assembly platform PulL and PulM in live bacteria. These components of the membrane assembly platform were localized in diffuse manner along the inner membrane. We would expect a similar distribution of PulI, PulJ or PulK. In addition, we suspect that the formation of the PulI-PulJ-PulK complex is of transient nature and that the closest that we get to a marker of an active assembly site is the pilus fiber itself.

Supplementary figure 1: An alternative procedure for labeling and detection of surface-exposed PulG is presented. Although very promising, a few negative controls are needed for the fluorescence microscopy. A strain not expressing PulG-BAP would provide specificity for the assay. Another control to account for the specificity of the labeling would be using ΔpulHIJK::pulG-+ pulHIJK, but omitting the addition of purified biotin ligase.

This part has been removed and experiments have been replaced with wild type PulG, as suggested by Reviewer 1.

2nd Editorial Decision 09 November 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-78128. It has now been seen again by referees 2 and 3, whose comments are enclosed below. As you will see, both referees find the manuscript substantially improved and are fully supportive of publication. Referee 2, however, does have a number of remaining concerns regarding presentational issues that I would ask you to address in a final revision of your manuscript.

Thanks also for alerting me to the recently published JBC paper: this does not in any way affect our assessment of your work, but given its relevance I think it would be important that you cite and at least briefly discuss the study.

I also have a couple of points from the editorial perspective:

- In the abstract, I notice a number of places where you seem to have used an unnecessary "the", and I would suggest you remove these. Specifically, line 21 "prime the pseudopilus assembly" should be "prime pseudopilus assembly"; line 22 "the secretin channel opening" should be "secretin channel opening"; line 24 "nucleate the filament assembly" should be "nucleate filament assembly". Can you fix this?

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide the original, uncropped and unprocessed scans of all gels used in the figures (or at least of key data panels)? These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. Ideally, we would ask for separate files for each figure panel, which you can upload as a single zip file as "source data" - via EJP. These will then appear as supplementary files and be directly linked to each relevant main figure. Please let me know if you have any questions about this policy.

Once we have received this final version of your manuscript, we should then be able to accept it for publication in the EMBO Journal.

REFEREE REPORTS

Referee #2:

The paper is much-improved and represents an important contribution to understanding Type II secretion are related processes. The paper is very dense and difficult to read in places due to its complexity, and yet there are still a number of details missing with respect to methods and strain descriptions. Below I have suggestions for clarifying/correcting some issues, listed as they appear in the text.

Are the pili in the immunofluorescence images bundles or does the fluorescence just increase their diameter so that they appear to be very thick - comparable to the cell diameter? If they are bundles, then it is the length of bundles, not individual filaments that is being measured. This should be clarified.

Figure legend S1 (B) "Secreted PulA immunodetection in WT and ΔpulH bacteria expressing low pul genes at near chromosomal levels in strain PAP5207." Do you mean low copy number? Low gene expression levels? Please clarify.

How were proteins quantified in Fig. 1A, 1F, 2D and S1C etc.? Densitometry? Add to methods/fig. legend.

For Figures 2 and 7 the figures are not presented in order. Either the panels should be rearranged or they should be discussed in the order they are presented. Say what background the complemented strains in 2B are (ΔpulHIJK).

Lines 135-138. "Although the ΔpulHIJK 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). Explain how pilus assembly efficiency is quantified. I assume you mean sheared PulG relative to total PulG? eg. ratio in lane 2/lane 1? For 1X or 10X? If so, the values shown in 2D do not appear to agree with those in 2C. For 1X, WT looks like ~100% (lane 14/13) whereas complemented (lane 2/1) looks like ~20%; for 10X, WT looks like ~200% whereas complemented looks like 30-40%.

Line 149. "PulI and PulJ together promoted efficient pilus assembly" add "relative to the complemented strain".

The green-labeled pili are very hard to see in many of the immunofluorescence images, even in those where they are abundant.

Line 165-167. "Taken together, our results suggest that the initiation of PulG filament assembly is impaired in the ΔpulHIJK mutant, but neither its elongation nor the secretin channel opening." Fix this sentence - perhaps add "are impaired". Also, the title for this section should read: "Minor pseudopilins are not required for opening of the secretin channel", as channel opening was not directly tested. Same goes for the sentence in line 367-368 of the Discussion - say the minor pilins "are not required ...".

Lines 170-171. This sentence suggests that "we" are Korotkov and Hol. Instead say something like "Based on the findings of Korotkov and Hol, we hypothesized that ..." or "... as was shown for the GspKIJ complex in ETEC."

Fig. 4F. Is this a Coomassie-stained gel? If not, what Abs are used for which blots?

Line 287. "..bringing the GspI β3-β4 loop close to the phospholipid headgroups" - show the β3-β4 loop in Fig. 4D. Also show PulJ:L16 and PulI:L10 in Fig. 5J and/or 6D (note: none of the figures are numbered).

Line 288. "Interestingly, a similar behavior has been observed ..." - modify: "a similar *in silico* behavior"

Line 305. Modify sentence to "Residue E35 of ETEC GspI (Fig. 7A) lies close to the interface of GspI ..." and reference the Korotkov/Hol 2008 paper.

Referee #3:

The authors have addressed all reviewers' comments. Their responses are satisfactory. They have quantified their data, measured the effect of single pseudopilin gene deletions and various pulI point mutations on pullulanase secretion, added new MD simulations, rewritten parts of the manuscript as well as reorganized it. The manuscript has improved significantly. I recommend it for publication.

2nd Revision - authors' response 10 November 2011

Referee #2:

The paper is much-improved and represents an important contribution to understanding Type II secretion are related processes. The paper is very dense and difficult to read in places due to its complexity, and yet there are still a number of details missing with respect to methods and strain descriptions. Below I have suggestions for clarifying/correcting some issues, listed as they appear in the text.

Are the pili in the immunofluorescence images bundles or does the fluorescence just increase their diameter so that they appear to be very thick - comparable to the cell diameter? If they are bundles, then it is the length of bundles, not individual filaments that is being measured. This should be clarified.

This is a question to which we do not have a firm answer at this point. In recent electron microscopy images we typically observe little bundling of PulG pili (Cisneros DA, Pehau-Arnaudet G and Francetic O, *manuscript in preparation*). This is in contrast to previously published studies, in which electron microscopy imaging was done by a different person and using different conditions (Vignon *et al.,* 2003, J. Bact.). For immunofluorescence, bacteria and pili are bound to poly-L-lysine, which may or may not cause bundling of PulG pili. On the other hand, pili appear the same regardless of whether they are visualized using quantum dots (as we showed in the first version of the manuscript) or using primary and secondary antibodies, excluding the possibility that antibodies may cause the bundling. Therefore, it is more likely that the brightness of the images leads to the apparent pili thickness.

Several factors can also affect the apparent pili thickness: 1) There are two “layers” of antibodies bound to each observed filament, therefore the apparent thickness is already altered. 2) Each fluorescent molecule emits light in all directions and from several planes, which is afterwards blurred, so the apparent thickness is also altered accordingly. 3) It is relatively standard nowadays to observe GFP molecules (~5-10 nm) or quantum dots (~20-50 nm) at the single molecule level using the same microscopy technique we use, where the pixel size is in the order of 30-100 nm and where the resolution limit is ~200 nm. Therefore it is difficult to assess the real thickness of the pili we observe.

Whichever the case, we do not think that this issue would have an influence on our conclusions. Even if the PulG pili were bundled, longer filaments would make longer bundles. (this would suggest an interesting possiblity of colocalization and coordination of several secretons). However, pili seem to behave as single filaments and their length correlates negatively with the assembly efficiency. Furthermore our immunofluorescence results correlate well with immunoblot analysis.

Figure legend S1 (B) "Secreted PulA immunodetection in WT and ΔpulH bacteria expressing low pul genes at near chromosomal levels in strain PAP5207." Do you mean low copy number? Low gene expression levels? Please clarify.

We apologize for this mistake which arose from multiple rounds of corrections and escaped our attention. Indeed, we meant to indicate low plasmid copy number. We changed the figure S1 legend to: “.....bacteria with reduced *pul* gene expression in strain PAP5207 (see main text)”

How were proteins quantified in Fig. 1A, 1F, 2D and S1C etc.? Densitometry? Add to methods/fig.

legend.

The calculation of PulG pilus assembly efficiency and PulA secretion efficiency is now described in Supplementary Materials and Methods.

For Figures 2 and 7 the figures are not presented in order. Either the panels should be rearranged or they should be discussed in the order they are presented. Say what background the complemented strains in 2B are (Δ pulHIJK).

The order of panels in Figure 2 has been changed as requested. The order of panels in Figure 7 has remained unaltered, as Fig. 7A is mentioned in the text at the beginning of this section. Legend to Fig. 2 has been changed accordingly (lines 758-768).

Lines 135-138. "Although the ΔpulHIJK 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). Explain how pilus assembly efficiency is quantified. I assume you mean sheared PulG relative to total PulG? eg. ratio in lane 2/lane 1? For 1X or 10X? If so, the values shown in 2D do not appear to agree with those in 2C. For 1X, WT looks like ~100% (lane 14/13) whereas complemented (lane 2/1) looks like ~20%; for 10X, WT looks like ~200% whereas complemented looks like 30-40%.

We introduced some changes in lines 134-138 to make the reader aware of the anomaly that the *ΔpulHIJK* mutant produces less PulG (and hence appears to assemble less pili).
"Expression of minor pseudopilin genes *pulHIJK in trans* under p_{lacZ} control (Fig. 2C, lane 2) restored pilus assembly, albeit not to WT levels. This is due to the fact that the *ΔpulHIJK* 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)." In this way, we think that the reader has all the elements to infer any possible caveats in this experiment.

The pilus assembly efficiency was calculated as ($PulG_{SF}$/($PulG_{SF}$ + $PulG_{cell}$). We chose for the quantification the 10X values and this was explained in the figure legend. For the 1X values we did not perform enough immunoblots to perform statistics in the time-frame of this revision. The 10X value also shows the difference between the complemented with *pulIK* and Vector, which we had shown in the first version of the manuscript. In any case, as stated before, we think that the relevant control for this experiment is the complemented strain. Besides, thanks to this reviewer, all the conclusions we make here are made with respect to the complemented *ΔpulHIJK* mutant (see comment below about line 149). One should also take into account that the *ΔpulHIJK* produces less PulG than WT and that the western blot saturation levels of antibodies could be different in the 1X and 10X, which could explain the differences found by the reviewer (although his/her numbers do not match ours because of the different formula used). However, we were careful to always compare the WT, the *ΔpulHIJK* mutant complemented with *pulHIJK* and *pullJ* in the same conditions. The main point that PulI and PulJ together promote pilus assembly to levels comparable to the complemented strain remains and is further supported by the mutational analysis (Fig. 7).

Line 149. "PulI and PulJ together promoted efficient pilus assembly" add "relative to the complemented strain".

This sentence was corrected as the reviewer rightfully suggested.

The green-labeled pili are very hard to see in many of the immunofluorescence images, even in those where they are abundant.

Brightness was enhanced equally in Figures 1 and 2 but only in panels where green pili are shown.

Line 165-167. "Taken together, our results suggest that the initiation of PulG filament assembly is impaired in the ΔpulHIJK mutant, but neither its elongation nor the secretin channel opening." Fix this sentence - perhaps add "are impaired". Also, the title for this section should read: "Minor pseudopilins are not required for opening of the secretin channel", as channel opening was not directly tested. Same goes for the sentence in line 367-368 of the Discussion - say the minor pilins "are not required ...".

We followed the reviewer's suggestion and added "are impaired" in lines 165-167 and " are not required" to line 367-368.

Lines 170-171. This sentence suggests that "we" are Korotkov and Hol. Instead say something like

"Based on the findings of Korotkov and Hol, we hypothesized that ..." or "... as was shown for the GspKIJ complex in ETEC."

The phrase now reads: “Since soluble versions of PulI and PulJ homologues were shown to be part of a complex (Korotkov and Hol, 2008), we hypothesized that the ability of PulI and PulJ to promote pilus assembly was related to their binding.”

Fig. 4F. Is this a Coomassie-stained gel? If not, what Abs are used for which blots?

In the previous version on the bottom of each panel we had indicated the antibody used (PulI, PulK etc.). To make this clear, the antibodies are now specified by adding the prefix “anti-” in this figure. The figure legend also specifies that this panel is an immunoblot.

Line 287. "..bringing the GspI β3-β4 loop close to the phospholipid headgroups" - show the β3-β4 loop in Fig. 4D. Also show PulJ:L16 and PulI:L10 in Fig. 5J and/or 6D (note: none of the figures are numbered).

The loop is now indicated in Figure 6D and snapshots of residues of GspI L10, GspJ L16 and GspK L10 are shown in Figure 5J. The legend of Fig 5 has been changed accordingly. Fig 6D is now mentioned in line 287.

Line 288. "Interestingly, a similar behavior has been observed ..." - modify: "a similar in silico behavior"

The sentence has been corrected as suggested.

Line 305. Modify sentence to "Residue E35 of ETEC GspI (Fig. 7A) lies close to the interface of GspI ..." and reference the Korotkov/Hol 2008 paper.

The sentence has been modified and the reference has been added.

