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The number of Neisseria meningitidis type IV pili determines host cell interaction

Anne-Flore Imhaus and Guillaume Duménil

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Transaction Report:

(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.)

Editor: David del Alamo

1st Editorial Decision 18 February 2014

Thank you for the submission of your manuscript entitled "A critical number of Neisseria meningitidis type IV pili is required for efficient interaction with host cells". We have now received the full set of reports from the referees that were asked to evaluate your study, which I copy below.

As you can see from their comments, all referees are rather positive and support the publication of your manuscript. In general, they are convinced that the evidence presented is conclusive, but still point out to some technical shortcomings that will need to be addressed. Although these concerns are explicitly mentioned in the referee reports and thus I will not repeat them here, I would like to draw your attention in particular to the comments of Referee #1, in line with point 2 of referee #3, regarding the subcellular localization of PilV and PilX.

Given these positive evaluations, I would like to invite you to submit a revised version of the manuscript. Please be aware that your revised manuscript must address the referees' concerns, experimentally if required, and their suggestions should be taken on board. In this regard, do not hesitate to contact me if you have any question, need any further input or anticipate any problems along the revision process.

It is 'The EMBO Journal' policy to allow a single round of revision only, which should be submitted within the next three months. As a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, we request that you contact me as soon as possible upon

publication of any related work in order to discuss how to proceed. Similarly, should you foresee a problem in meeting the three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, 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 initiative, please visit our website: http://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

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

REFEREE REPORTS:

Referee #1:

In this manuscript the authors focus on an analysis of the function of the so-called minor pilins in the type IV pili system of Neisseria meningitidis. Focusing on the minor pilins PilV and PilX it is reported that (i) PilX-Flag and PilV-Flag are not detected in the pilus fibers but only in the periplasm; (ii) PilX and PilV are important for pilus fiber biogenesis (and not in retraction); (iii) PilX and PilV exert their function in the periplasm; (iv) manipulating the number of pilus fibers per cell appropriately phenocopies the pilX and pilV mutants. Based on these findings it is suggested that PilX and PilV are involved in the initiation phase of pilus fiber formation and they function in the periplasm.

It is well-known that lack of minor pilins causes a reduction in the number of pilus fibers. Also, it has been suggested that PilX may function as a retraction antagonist. More alarming, it has previously been suggested that PilX is involved in a process that allow the pilus fiber to undergo conformational changes in response to external force suggesting that PilX is part of the pilus fibers. Finally, in Pseudomonas aeruginosa as well as in N. gonorrhoeae minor subunits have been reported to be present in assembled pilus fibers. It is not clear how the findings reported in the manuscript can be easily reconciled with the previous findings.

There are quite a number of typos in the manuscript (genes in italics and proteins starting with capital).

I have several comments that I would like to see addressed experimentally.

Comments:

1. line 134-158: What are the accumulation levels of PilX-Flag and PilV-Flag in comparison to PilX and PilV in the WT strain? It is essential to clarify this because several of the experiments address the ratio between PilX-Flag and PilV-Flag in the pilus fibers and the periplasm. If the two proteins are overexpressed, then the data reported do not reflect the WT situation. Also, the functionality of PilX-Flag and PilV-Flag is not clear. The two proteins should be expressed at native levels to make conclusions about functionality.
2. line 141-142: Most of the pilus fibers in Fig. 1A do not seem to be connected to cells. And vice versa, most cells do not have pilus fibers. Is this also observed by immunofluorescence with anti-PilE antibodies? This is not a criticism but more a question of clarification for this reviewer.
3. line 144-145: The inability to detect PilX-Flag and PilV-Flag in the pilus fibers is a negative result and as such one should be careful not to make too many conclusions based on this experiment. An important question is what the detection limit of the method is. If PilX and PilV are only present at the tip of the fibers or in low amounts along the length of the fibers, it may not be possible to detect it by immunofluorescence. Given that PilX and PilV are indeed detected in bulk pilus fibers (Fig. 1DE), the authors should try to detect PilX-Flag and PilV-Flag using immuno-gold staining. It is essential to clarify this because many conclusions are based on the lack of detection of PilX-Flag and PilV-Flag in the pilus fibers.
4. line 184: where is it shown that PilV is overexpressed?

5. line 186: Is PilX also overexpressed?
6. line 198-206: It has previously been suggested that PilX may function as an antagonist of retractions. If PilV and PilX are only involved in extension of pilus fibers, then the prediction would be that in a pilT strain (which cannot retract due to lack of the retraction ATPase PilT), the pilus phenotype should be as in WT.
7. line 226-238: Are PilX-mCherry or PilV-mCherry detected in pilus fibers?
8. line 248-254: Are the PilV and PilX E5A variants detected in pilus fibers?
9. line 586-604: The difference between minor pilins and pseudopilus should be better described.
10. Through-out the text: PilX and PilV are referred to as periplasmic proteins. Maybe it would be more correct to say that they are integral inner membrane proteins with a large domain in the periplasm.

Referee #2:

The manuscript by Imhaus and Dumenil shows that deletion of the minor pilins PilV and PilX from Neisseria meningitidis modestly affects pilus levels but these changes have significant effects on pilus functions. They provide compelling evidence that both proteins act within the periplasm, contrary to what has been proposed for these and other minor pilins. The manuscript is well-written, the experiments are for the most part well-executed and the results are intriguing and contribute to our understanding of these enigmatic proteins in pilus biogenesis. I have listed comments/criticisms below, listed in the order they appear in the text.

Title/Abstract. Although the title is grammatically correct it seems like it should say "A critical number of type IV pili are required ...". I recommend a different title altogether, which focuses on the minor pilins, their localization and their effects on piliation. For instance, "Neisseria meningitidis minor proteins PilV and PilX act in the periplasm to initiate pilus assembly". I feel that the quantification of pili was somewhat over-interpreted and should not be emphasized in the title, nor in the last line of the Abstract, which states: "We show that specific type IV pili dependent functions require different ranges of pili numbers: at least one for competence, two for adhesion and aggregation and three to five for crosstalk with host cells."

Introduction. Say something regarding the PilV and PilX proteins - where they are coded in the genome relative to PilE, other pilus genes, their protein size relative to PilE, how similar they are in sequence to PilE and to each other, etc..

Line 152-154. State in the Results what cells are used for the recruitment and adhesion assays. Fig. 1B. "Recruitment" is not very descriptive - call the Y-axis "Ezrin recruitment" or "Cortical plaque formation". Line 438 of Methods - indicate that the cells are HUVECs.

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Line 183. Note that measuring OD values does not necessarily ensure that the same number of cells is added to each well when the bacteria are aggregating. This is especially a problem when some strains aggregate more than others. Aggregation can be compounded by dilution of the bacteria in PBS, which can induce pilus:pilus interactions. The cells in the ELISA plate wells should be quantified (in a relative manner) using an antibody against an unrelated surface marker and piliation levels normalized to control for variations in cell levels. This is true for all of the ELISA-based piliation data. Why were cells dried on plates and fixed? Why not look at intact unfixed cells to see pili on surface?

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The model shown in Fig. 7 is nice but its not clear why aggregation shouldn't provide a critical number of pili, as pili from adjacent cells could contribute to adhesion and signaling of the host cell. This effect is ignored in the figure by only showing the 5 pili on the central cell in panel iv, and none on the adjacent cells in the aggregate.

Other points.

The paper is not referenced rigorously. For instance, the Giltner et al. review referenced on line 48 should be replaced with that of one that first recognized the similarity between type IV pili and type II secretion systems (eg. Pugsley); the pilus structure reference on Line 75 should be updated; Giltner et al., JMB 2010 should be included in line 165.

The order of the sections is odd. Why is Materials and Methods between Results and Discussion, and Acknowledgements is after the Figure Legends?

Present V then X consistently (Fig. 2C, 5B, 5C show X then V)

Fig. 5C is mislabeled - X-axis should indicate that the G-1N variants are expressed in a wild type background, not in the mutant background, as shown in 5B. B and C should be aligned vertically.

The Supplementary figure headers (bottom right) are inconsistent and there are two Fig. S3s.

Referee #3:

This manuscript describes some interesting experiments aimed at investigating the role of two pilin-like proteins, PilX and PilV, in type IV pilus (TFP) biogenesis and function. Both proteins have pilus-like structural features and their mutation, unlike other minor pilins, does not completely ablate pilus formation. Rather, they modulate specific TFP function: for example, pilX mutants fail to demonstrate aggregation. This presents a puzzle: how do proteins which ostensibly constitute a minor component of the TFP fiber have such a profound effect on function, but a much lesser effect on piliation? The authors advance a plausible and interesting hypothesis, that PilX and PilV exert their effects predominately by modulating the average number of pili per cell. The heart of the paper is the elegant experiment in Fig 6, where piliation is modulated by induction of increasing levels of the assembly ATPase, PilF, and the phenotypic effects of the pilX and pilV mutations can be effectively reproduced by matching the corresponding levels of piliation in those mutants. This is nicely done, and makes the point well. There were, however, a number of details surrounding the arguments presented in the paper which need to be addressed.

Major
1. The authors state at several points in the manuscript that PilX and PilV are required for efficient

initiation of TFP formation. What is the evidence that they play a role in initiation specifically? The authors' data are broadly in agreement with previously published observations- the pilX and pilV mutations lead to reduced piliation of 27 and 61% respectively. There is also data presented to show that both proteins appear to remain in the periplasm and require cleavage by PilD. But this does not, of itself, provide sufficient evidence to establish unequivocally that PilX and PilV are involved in initiation of pilus formation.

2. An experimental approach was adopted to demonstrate that PilX and PilV function in the periplasm, by coupling each protein to mCherry, on the basis that this much more bulky protein would be prevented from crossing the outer membrane. The reasoning is a little sketchy here- the authors cite recent experimental work on the secretins, which mediate TFP passage across the outer membrane, but the situation is not as straightforward as they suggest. The prevailing model for secretin function is that they form gated pores; the majority of structural studies have, however, been conducted on purified secretins which are in the 'closed' state. With the exception of the type III secretion system assembly, we really know very little about the dimensions of the pore during the secretion process. In any case, it seems plausible that the pore measures at least as far as the diameter of a type IV pilus i.e. about 6nm. Is it really clear that fusion with mCherry (3x5nm, see line 577) would prevent secretion? Layered on top of this is the argument that PilX and PilV are not incorporated into the pilus fiber anyway, even in their native state (Fig 1). The mCherry fusion therefore seems to be designed to prevent PilX/PilV secretion which doesn't happen anyway, and therefore it is impossible to tell whether it has worked or not. This whole argument seems confused and, at the very least, requires careful re-writing and improved presentation.

3. Lines 159-174: band intensities from Western blots are notoriously non-linear with protein concentration. What steps were taken to ensure linearity of signal here with protein concentration, to ensure there are no systematic errors in the estimated percentages reported on line 171?

Minor
1. Some parts of the paper could be better written and presented- it would benefit from more thorough proof-reading. In addition to obvious typographical errors (eg line 188), the Abstract needs modification to better convey the central hypothesis and the methods used to support it.

2. Line 225: is this figure relative to a value of 1.0 for wild type? This is not clear as it is currently presented in the text.

3. The text needs to be checked throughout for consistent uses of 'pilus' (singular) and 'pili' (plural).

1st Revision - authors' response 21 March 2014

Response to reviewers – Imhaus et al.

(Underlined sections indicate where additional experiments were provided)

Referee #1:

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It is well-known that lack of minor pilins causes a reduction in the number of pilus fibres. Also, it has been suggested that PilX may function as a retraction antagonist. More alarming, it has previously been suggested that PilX is involved in a process that allow the pilus fibre to undergo conformational changes in response to external force suggesting that PilX is part of the pilus fibres. Finally, in Pseudomonas aeruginosa as well as in N. gonorrhoeae minor subunits have been reported to be present in assembled pilus fibres. It is not clear how the findings reported in the manuscript can be easily reconciled with the previous findings.

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Comments:

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The experiment that quantitatively addresses the question of the ratio of extracellular pilus localization vs periplasm is the pilus preparation experiments depicted in figure 1E-F. This experiment is done on WT cells.

To strengthen this point with a different technical approach we have also used an imaging strategy. Flag-tagged PilV and PilX were expressed under the control of the lac promoter and their localization determined using immunofluorescence. This staining shows a periplasmic staining and no evidence of pilus localization. Experiments depicted in the initial manuscript used the maximum expression level (1 mM IPTG) to maximize the chances to detect a potential pilus localization.

We now have repeated these experiments at lower level of inducer mimicking the endogenous level of expression (25 µM IPTG) with the same result (Figure 1A).

The functionality of the PilX-Flag and PilV-Flag proteins have now been evaluated at different levels of inducer and compared with the wild type proteins (Figure 1C-D). At all the concentrations of inducer tested the presence of the Flag in PilX had no effect on its ability to promote adhesion and the Flag in PilV had no effect on its ability to promote plasma membrane reorganization. Figures 1C and D have been changed accordingly.

2. line 141-142: Most of the pilus fibres in Fig. 1A do not seem to be connected to cells. And vice versa, most cells do not have pilus fibres. Is this also observed by immunofluorescence with anti-PilE antibodies? This is not a criticism but more a question of clarification for this reviewer.

Absence of pili or pili with no obvious link to bacterial bodies are frequently observed in such conditions (e.g. Giorgiadou and Pelicic Mol. Microbiol. 84(5) 2012 fig5B).

We have now included additional panels in figure 1A. In particular we added a Flag staining on the

*pilEpilE*Flag strain (deleted endogenous gene and complemented with the PilE-Flag). In these conditions pili labelling is more continuous.

3. line 144-145: The inability to detect PilX-Flag and PilV-Flag in the pilus fibres is a negative result and as such one should be careful not to make too many conclusions based on this experiment. An important question is what the detection limit of the method is. If PilX and PilV are only present at the tip of the fibres or in low amounts along the length of the fibres, it may not be possible to detect it by immunofluorescence. Given that PilX and PilV are indeed detected in bulk pilus fibres (Fig. 1DE), the authors should try to detect PilX-Flag and PilV-Flag using immuno-gold staining. It is essential to clarify this because many conclusions are based on the lack of detection of PilX-Flag and PilV-Flag in the pilus fibres.

Although absence of staining is a negative result we have been very careful in providing adapted controls for this experiment. The question is the following: if PilX or PilV were displayed along the pilus fibre at low amounts could we detect them?

To mimic this situation we have generated a strain (*pilE* Flag) that co-expresses PilE-Flag at different levels using the lac promoter in addition to the endogeneous *pilE* gene under it own promoter (stronger than lac). This results in a "hybrid pilus", mostly with the non-labelled PilE and with a regulatable amount of the flagged protein. In the initial version of the manuscript only one concentration was shown (1 mM). The result is a dotted staining along pili fibres. One dot corresponds to one PilE-Flag among non-tagged PilE, or perhaps areas where the PilE-Flag proteins are more concentrated.

We now have added lower concentrations of inducer to mimic the expression level of PilV and PilX, or even lower (10 µM IPTG). In these conditions the PilEFlag protein are easily detectable along pilus fibres (Figure 1B). We believe this convincingly shows that we are technically able to detect a Flag tagged protein expressed at a low level along the pilus fibre.

In the same conditions, same promoter, same amount of inducer, same tag and antibody used for detection PilX-Flag and PilV-Flag are never observed along the fibre. Furthermore, overexpression of PilX-Flag and PilV-Flag did not provide any signal either. This section has been extensively rewritten to clarify.

4. line 184: where is it shown that PilV is overexpressed?

Supplementary figure S1 shows the level of expression of PilV as a function of inducer.

5. line 186: Is PilX also overexpressed?

Yes, same as above

6. line 198-206: It has previously been suggested that PilX may function as an antagonist of retractions. If PilV and PilX are only involved in extension of pilus fibres, then the prediction would be that in a ΔpilT strain (which cannot retract due to lack of the retraction ATPase PilT), the pilus phenotype should be as in WT.

Relative to the WT strain the *pilT* strain has about 6 times more pili on its surface (our unpublished results). Double mutants *pilXpilT* or *pilVpilT* show more pili than wild type but a bit less than *pilT* (4-5 times more than the wild type). This is compatible with a reduced level of initiation.

7. line 226-238: Are PilX-mCherry or PilV-mCherry detected in pilus fibres?

This information is indicated in line 233, “The mCherry fusion constructs with PilE, PilV and PilX all localized in the bacterial periplasm and, as expected, no evidence of organization as pilus fibres could be seen (Figure 3A).”

8. line 248-254: Are the PilV and PilX E5A variants detected in pilus fibres?

This has not been tested.

9. line 586-604: The difference between minor pilins and pseudopilus should be better described.

The term pseudopilin is typically used for GspH-K in type II secretion and by extension occasionally used for the homologs in type IV pili, PilH-K. To avoid confusion the term “pseudopilin” was replaced by “pilin-like proteins”.

10. Through-out the text: PilX and PilV are referred to as periplasmic proteins. Maybe it would be more correct to say that they are integral inner membrane proteins with a large domain in the periplasm.

The exact location inside the periplasm remains to be determined. It is likely that PilX and PilV are mostly associated with the inner membrane although it is also possible that a certain proportion participate in the piliation machinery complex. The discussion has been modified accordingly (L401-404).

Referee #2:

The manuscript by Imhaus and Dumenil shows that deletion of the minor pilins PilV and PilX from Neisseria meningitidis modestly affects pilus levels but these changes have significant effects on pilus functions. They provide compelling evidence that both proteins act within the periplasm, contrary to what has been proposed for these and other minor pilins. The manuscript is well-written, the experiments are for the most part well-executed and the results are intriguing and contribute to our understanding of these enigmatic proteins in pilus biogenesis. I have listed comments/criticisms below, listed in the order they appear in the text.

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The abstract has been modified accordingly. We decided to maintain the original title, however, as we feel that the functional importance of the number of pili expressed by bacteria is the main conclusion of the manuscript. The role of PilV and PilX in pilus biogenesis is mentioned in the running title.

Introduction. Say something regarding the PilV and PilX proteins - where they are coded in the genome relative to PilE, other pilus genes, their protein size relative to PilE, how similar they are in sequence to PilE and to each other, etc..

This is now included (L93-96 and L433-436).

Line 152-154. State in the Results what cells are used for the recruitment and adhesion assays. Fig. 1B. "Recruitment" is not very descriptive - call the Y-axis "Ezrin recruitment" or "Cortical plaque formation". Line 438 of Methods - indicate that the cells are HUVECs.

This is now included

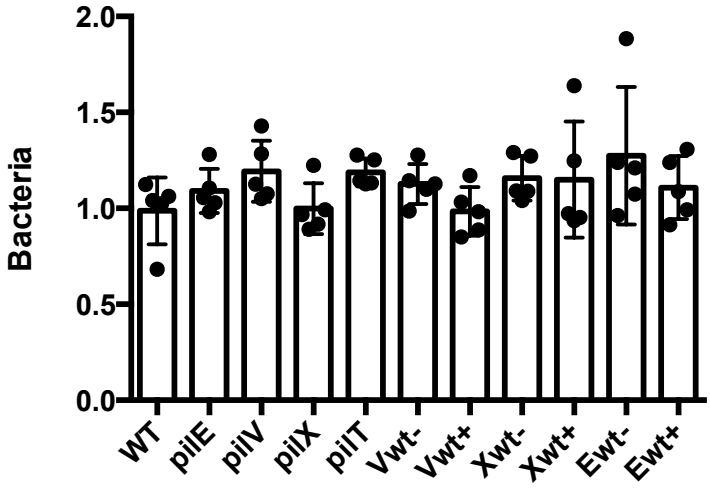
Line 159-174. State in the Results section that the results of Figs. 1D and 1E are from wild type cells (i.e. cells not over-expressing the minor pilins).

This is now included

Line 183. Note that measuring OD values does not necessarily ensure that the same number of cells is added to each well when the bacteria are aggregating. This is especially a problem when some strains aggregate more than others. Aggregation can be compounded by dilution of the bacteria in PBS, which can induce pilus:pilus interactions. The cells in the ELISA plate wells should be quantified (in a relative manner) using an antibody against an unrelated surface marker and piliation levels normalized to control for variations in cell levels. This is true for all of the ELISA-based piliation data. Why were cells dried on plates and fixed? Why not look at intact unfixed cells to see pili on surface?

The protocol used here is optimized to limit this kind of issue. The bacterial suspension is spun down in the well and the supernatant is collected carefully to avoid disturbing bacteria in the bottom, then the remaining liquid containing all the bacteria is allowed to dry to ensure that all bacteria are immobilized on the bottom, independently of their aggregative properties. The fixation step is to avoid any detachment during the procedure, which requires extensive washing steps (as well as ensuring that all bacteria are dead and allowing manipulation in a standard security level lab). Pili are highly dynamic, we would predict that staining of live cells would influence the piliation level, by preventing retraction for instance.

To control the amount of bacteria plated in the ELISA plates, an antibody directed against whole bacteria has been used. Results indicated below show that the same amount of bacteria is plated, independently of the aggregation phenotype of the strain. One-way ANOVA and Tuckey multiple comparison test indicate that the different values are not statistically different.



Line 188. "... had a synergistic effect"

This is now modified (L197).

Lines 191-195. Specify that anti-PilE antibodies were used to visualize the pili by immunofluorescence. Based on the images shown in Fig. 2D it is not obvious how many pili are present on a given cell. Thus, one certainly cannot quantify the number of pili to 3 significant figures. Are these measurements affected by aggregation of the bacteria?

Results presented in figure 2D aim to provide representative images. Results in figure 2 E-H are compiled from the observation of 150 individual bacteria for each strain.

The protocol optimized for “pilus counting” is different from the one used to generated images like in figure 1A-B. These stainings are performed at low concentrations of bacteria to obtain individual bacteria. Any bacterium involved in an aggregate was not counted.

Line 240-254. Were intermediate IPTG concentrations investigated? The high expression levels at 1 mM IPTG (Fig. S2) may allow incorporation of the E5A mutants into pili even if this interaction is less favoured. The experiment should be repeated using IPTG concentrations that give wild type levels of PilV and PilX expression before concluding that the minor pilins do not need E5 and do not assemble into pili.

Different amounts of IPTG were used to test the functionality of *pilVE5A* and *pilXE5A* (Figure 4B-C). We feel these functional assays are more sensitive and demonstrative compared to the analysis of piliation. At wild type level of PilX and PilV the E5A mutants are perfectly functional.

Line 271-272. Clarify what is meant by "tight association of PilV and PilX with the piliation machinery". Do you mean a physical association? I'm not sure that the prepilin peptidase has been shown to physically associate with the piliation machinery.

The text has been modified to clarify. We meant functional interaction as there is currently no evidence for physical interaction (L288).

Line 295 -299. Provide more detail regarding quantification of aggregation. Numbers of aggregates? Numbers of bacterial in aggregates vs. free bacteria?

This is now clarified in the text. Graphs indicate the numbers of bacteria in aggregates vs. total bacteria (L311-316).

The model shown in Fig. 7 is nice but its not clear why aggregation shouldn't provide a critical number of pili, as pili from adjacent cells could contribute to adhesion and signalling of the host cell. This effect is ignored in the figure by only showing the 5 pili on the central cell in panel iv, and none on the adjacent cells in the aggregate.

The text and figure have been modified to include this important point (L458-464).

Other points.

The paper is not referenced rigorously. For instance, the Giltner et al. review referenced on line 48 should be replaced with that of one that first recognized the similarity between type IV pili and type II secretion systems (e.g. Pugsley); the pilus structure reference on Line 75 should be updated; Giltner et al., JMB 2010 should be included in line 165.

The correct references are now included.

The order of the sections is odd. Why is Materials and Methods between Results and Discussion, and Acknowledgements is after the Figure Legends?

This is now corrected.

Present V then X consistently (Fig. 2C, 5B, 5C show X then V)

This is now corrected.

Fig. 5C is mislabelled - X-axis should indicate that the G-1N variants are expressed in a wild type background, not in the mutant background, as shown in 5B. B and C should be aligned vertically.

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Major

1. The authors state at several points in the manuscript that PilX and PilV are required for efficient initiation of TFP formation. What is the evidence that they play a role in initiation specifically? The authors' data are broadly in agreement with previously published observations- the pilX and pilV mutations lead to reduced piliation of 27 and 61% respectively. There is also data presented to show that both proteins appear to remain in the periplasm and require cleavage by PilD. But this does not, of itself, provide sufficient evidence to establish unequivocally that PilX and PilV are involved in initiation of pilus formation.

The main argument for a role in initiation comes from the determination of the number and length of the pili in the mutants (Figure 2E-H). *pilX* and *pilV* strains express a lower number of pili that are the same length as the wild type strain. If they played a role in extension the prediction would be that they would express the same number of pili but shorter. If the PilX and PilV proteins slowed down retraction, the pili in mutants would retract faster and thus be shorter as well on average. Furthermore, in this last scenario motility due to retraction would be expected to be faster which is not the case as shown in figure 2J. The results section describing figure 2 as well as the discussion have been modified to clarify this important point (L200-221).

2. An experimental approach was adopted to demonstrate that PilX and PilV function in the periplasm, by coupling each protein to mCherry, on the basis that this much more bulky protein would be prevented from crossing the outer membrane. The reasoning is a little sketchy here- the authors cite recent experimental work on the secretins, which mediate TFP passage across the outer membrane, but the situation is not as straightforward as they suggest. The prevailing model for secretin function is that they form gated pores; the majority of structural studies have, however, been conducted on purified secretins which are in the 'closed' state. With the exception of the type III secretion system assembly, we really know very little about the dimensions of the pore during the secretion process. In any case, it seems plausible that the pore measures at least as far as the diameter of a type IV pilus i.e. about 6nm. Is it really clear that fusion with mCherry (3x5nm, see line 577) would prevent secretion?

As noted by this reviewer the exact size of the open secretin is not yet clear. We feel it is relatively safe to suggest, however, that insertion of the mCherry tag that would roughly double the size of the pilus would block secretion. Furthermore, we provide experimental evidence for this in figure 3B, when the mCherry protein is fused to PilE in the *pilE* background, bacteria are unable to produce any pili. In addition, when the *pilEmCherry* construct is expressed in the wild type background a dominant negative effect is observed on piliation (not shown).

Layered on top of this is the argument that PilX and PilV are not incorporated into the pilus fibre anyway, even in their native state (Fig 1). The mCherry fusion therefore seems to be designed to prevent PilX/PilV secretion which doesn't happen anyway, and therefore it is impossible to tell whether it has worked or not. This whole argument seems confused and, at the very least, requires careful re-writing and improved presentation.

The results presented in figure 1 argue that the vast majority of the PilV and PilX are localized in the periplasm. They cannot exclude, however, that a small, but functionally important, proportion of the protein is localized in the pilus fibre. The objective with the mCherry fusion is to show that PilX and PilV exert their functions in the periplasm. Although this might sound redundant this point is important because the common hypothesis presented on most manuscripts on this topic describe PilX and PilV as carrying out their function inside the pilus fibre (e.g. Helaine and Forest, PNAS, 104 (40) 2007).

3. Lines 159-174: band intensities from Western blots are notoriously non-linear with protein

concentration. What steps were taken to ensure linearity of signal here with protein concentration, to ensure there are no systematic errors in the estimated percentages reported on line 171?

These experiments were performed using an ImageQuant LAS400 digital imaging system that allows direct acquisition of luminescence signals on a blot. This device allows quantitative measurements with much more linearity than band analysis on film for instance. As presented on figure 1E three dilutions of the pilus preparation were used for each protein. In all cases we obtained a linear relationship between the loaded amount and the signal with the procedure used.

Minor

1. Some parts of the paper could be better written and presented- it would benefit from more thorough proof-reading. In addition to obvious typographical errors (e.g. line 188), the Abstract needs modification to better convey the central hypothesis and the methods used to support it.

The manuscript has been proofread and corrected, the abstract in particular.

2. Line 225: is this figure relative to a value of 1.0 for wild type? This is not clear as it is currently presented in the text.

This point is clarified in the text (L240).

3. The text needs to be checked throughout for consistent uses of 'pilus' (singular) and 'pili' (plural).

This is now corrected.

2nd Editorial Decision 15 April 2014

I am pleased to inform you that your manuscript has been accepted for publication. Please see below the final comments from the referees, where they concur on the suitability of your study to The EMBO Journal.

Thank you for your contribution to The EMBO Journal and congratulations on a successful publication.

REFEREE REPORTS:

Referee #1:

This is a revised version of a manuscript that I have previously seen. In the manuscript the authors focus on an analysis of the function of the so-called minor pilins in the type IV pili system of Neisseria meningitidis. Focusing on the minor pilins PilV and PilX it is reported that (i) PilX-Flag and PilV-Flag are not detected in the pilus fibers but only in the periplasm; (ii) PilX and PilV are important for pilus fiber biogenesis (and not in retraction); (iii) PilX and PilV exert their function in the periplasm; (iv) manipulating the number of pilus fibers per cell appropriately phenocopies the pilX and pilV mutants. Based on these findings it is suggested that PilX and PilV are involved in the initiation phase of pilus fiber formation and they function in the periplasm.
All my comments on the original manuscript have been satisfactorily addressed in the revised version. In total, this manuscript presents highly interesting findings and new insights into the function of PilX and PilV in the type IV pili system.

Referee #3:

As detailed in my previous report, I consider this manuscript to be an important contribution to the field. The role of minor pilins has been much debated in recent years, and this work provides important new information on their function. I still think there is more work to be done to establish definitively the role of PilX and PIlV in initiation of pilus biogenesis, but that is beyond the scope of this work. The authors have responded well to the points raised and eliminated many of the typographical and grammatical errors in the original manuscript.