THE
EMBO
JOURNAL

Deep mutational scanning of the *Neisseria meningitis* major pilin reveals the importance of pilus tip-mediated adhesion

Paul Kennouche, Arthur Charles-Orszag, Daiki Nishiguchi, Sylvie Goussard, Anne-Flore Imhaus, Mathieu Dupré, Julia Chamot-Rooke, Guillaume Duménil

Review timeline:

Submission date:	29th Mar 2019
Editorial Decision:	26th Apr 2019
Revision received:	11th Jul 2019
Editorial Decision:	6th Aug 2019
Revision received:	10th Sep 2019
Accepted:	12th Sep 2019

Editor: Ieva Gailite

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.)

1st Editorial Decision 26th Apr 2019

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the reports, all reviewers express interest in the presented detailed mutational analysis of N. meningitidis major pilin PilE, and they appreciate the extent of the analysis and the quality of the data. However, they also raise a number of concerns that would need to be addressed before they can support publication of the manuscript.

From my side, I judge the referee comments to be generally reasonable, therefore I would like to invite you to submit a revised manuscript addressing the concerns of all reviewers. From the editorial side, I would like to ask you to add a paragraph in the discussion to place the findings in the broader context of the existing knowledge of adhesion and aggregation mediation by other types of pili. Please note that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

REFEREE REPORTS:

Referee #1:

For the manuscript by Kennouche et al. the authors generate a library of N. meningitidis point mutants covering the length of the major pilin, PilE. The library was analyzed for pilus production, pilus-mediated aggregation and adhesion to HUVEC using a clever approach that isolated pilus-producing bacteria (by FACS), aggregation-competent bacteria (by filtering) and adherent cells (by binding) and then subjected both the isolated functional mutants and the input mutants to next generation sequencing and compared the frequency of the mutants in these populations to identify residues involved in each of these three functions. Interesting mutants were generated de novo and analyzed further. They report residues in the N-terminus of the pilin that, when substituted, result in

short but more abundant pili, residues around a protruding and exposed lysine on the pilus surface that are involved in aggregation, and, most interestingly, residues at the tip of the pilin that appear to mediate adhesion. The approach is sophisticated and powerful, the analysis is rigorous and the data are beautifully represented. I have only minor concerns, listed below.

The statement in the abstract "Here, we simultaneously determined the regions of PilE involved in pili display, auto-aggregation and adhesion by using deep mutational scanning and mining this extensive functional map." This is somewhat overstated. Specific residues involved in these processes were identified but the results are not exhaustive. Additional regions involved in these processes might have been missed as single amino acid changes in these regions may not disrupt function.

N-terminal mutants: The authors show that the N-terminal mutants do not aggregate and state that deletion of the retraction ATPase "was sufficient to increase piliation of individual bacteria and restore aggregation in mutants with short pili" but they only report aggregation for one of the N-terminal/pilT double mutants and show no pilus images. What does "increase piliation" mean? Longer pili? Because they report that the mutants are already more piliated than WT.

The data in Fig. EV2E showing adhesion for the N-terminal mutants are important as they show that the short pili are functional, yet they are not cited until much later in the paper (line 203-205).

Lys140 region mutants: Mutations encoding 3 basic residues, K103, K144 and H149, disrupt aggregation but not piliation, and E99 mutants increase aggregation. What about K140? Was it not selected as piliated in the library screen because the antibody, 20D9, does not bind well to K140 mutants? E99 mutants are mentioned here as increasing aggregation but no results are shown for de novo E99 mutants. Was this phenotype not borne out?

Discussion: The results showing the involvement of tip-associated residues in adhesion is intriguing. However the Discussion does not adequately address the data in the literature supporting the presence of minor pilins at the pilus tip, which would block any interactions between the major pilin tips and host cell receptors. Could the tip-labeled pili represent broken pili that have their major pilins exposed, whereas the pili bound to cells are bound via their intact minor pilins? This is a controversial and poorly understood aspect of pilus biology that must be more carefully addressed.

There are several places where the authors refer to supplementary materials that were not made available: "mutagenic primer listed in Supplementary file 1", "Supplementary Table 1", "Supplementary Table 2", "Video 1", "supplementary information".

Line 110-112. "... the mutation scores corresponding to auto-aggregation were normalized by the mutation scores corresponding to piliation". I don't understand this scoring. The Y-axis reports a ratio of aggregation/piliation, which should not result in negative numbers. In addition, the data should be skewed to mutants that are piliated but do not aggregate (<1), with few mutants that are not piliated but aggregate (>1).

Line 130-131. Show the Western blot in addition to the band density plot.

Line 163-164. Something is missing here - commas? parentheses?

Line 168-172: Provide more explanation regarding these analyses including whether pili were sheared from the cells and purified.

Line 730, 744. "Dotted" should be "solid".

Line 759. "Purple" should be "blue".

The reference formatting is inconsistent in the bibliography.

Fig. 3C. What is all the background for the 20D09 Ab binding to K140Q?

Referee #2:

This interesting manuscript describes well-executed and beautifully illustrated studies aimed at distinguishing residues of the Neisseria meningitidis major pilin protein, PilE, that are critical for particular emergent functions of its type IV pilus filaments. These questions are challenging to address due to the multifunctional nature of the system. The authors used saturating mutagenesis of PilE followed by phenotypic assays and deep sequencing to delineate which aspects of the pilin contribute to each trait. They specifically queried adhesion to human cells, aggregation (pilus-pilus interactions), and piliation - an indirect measure of assembly/disassembly capacity.

I remain unconvinced that pili are adhering through a single PilE tip-exposed epitope alone rather than through a combination of this epitope and minor components. Single mutants of pilV or pilX still make pili with the other gene product.

Some commentary from an evolutionary perspective on the fact that most substitutions had little effect on the phenotypes of interest is warranted in the light of the statement on line 59-60 that there is an unusual number of constraints. Does this result imply that the pilin is able to tolerate significant sequence variation at non-critical positions while maintaining function? How do they converge on optimal sequences that maximize multiple functions? When phase variation occurs, are the critical residues maintained?

Other comments:

Line 35, carried should be mediated

Line 64, Neisseria pili also bind to abiotic surfaces, as shown by the adherence assays using purified pili. How would those interactions differ from those with host cell surfaces?

Line 88, what is the epitope recognized by this mAb? Line 152 says the authors 'suspected' its epitope was around residue 140 but do not say why. What does the nanobody recognize?

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Line 149-50, how conserved is this feature among Neisseria? What about other species that have pili that form bundles eg. Vibrio or E. coli?

Line 169, presumably this was binary (yes/no) and not quantitative?

Line 183, pilin, not pilus? The epitope is exposed in pili that are under tension - would that be a factor here?

Line 191, this statement seems contradictory - the authors say that they chose the 30 min timepoint to minimize the contribution of aggregation. Why would adherence defects correlate with aggregation defects if pilus length is key for the latter, unless both parameters rely on the expression of a minimal number of pili?

Line 199-201, if the authors performed flow with bacteria stained with mAb SM1, would they not be able to test the idea that pilus tip numbers per cell was important?

Line 233, the persistence length stated here is for labeled pili. What is the role of the flexible cell surface to which the pili are attached in this experiment?

Line 241, what is the secondary adhesive component in this scenario, and were there mutants in the authors' collection that could not perform this secondary adhesion step?

Line 249-50, the work on antibody-mediated inhibition of Haemophilus T4P binding to the host in should be cited here. PMID:25597921

Line 270, in addition to - this is part of assembly, not in addition to assembly

Line 292 -302, the authors often use pilin and pilus interchangeably throughout, which is not accurate. The mutational analysis was on one pilin protein, and the altered sequence may be exposed only in certain regions of the pilus. The potential role of minor pilins, which are present in these pili according to the authors own Western data, must be considered. The SM1 antibody also binds to stretched pili which have the epitope exposed under tension as may occur during adhesion. There is structural evidence from the Jensen lab, corroborated by genetic data from the Burrows lab, that the minor pilins form a complex that primes assembly and thus becomes part of the pilus tip. In the absence of more direct evidence that Neisseria is different, it would be worth keeping an open mind as to the configuration of the pilus tip.

Line 311-317, the pili imaged here are labeled, which could affect their persistence length and behavior. The persistence length of 5 microns for P. aeruginosa pili was likewise estimated from thermal fluctuations of labeled pili so may not be accurate. New work by the Persat lab on unlabeled pili using iSCAT imaging may lead to more accurate estimates.

Line 331, what is 'unassisted' flow cytometry?

Line 334, this hypothesis could be tested using retraction deficient versions of these mutants in combination with the point mutants of PilE that appear to have adhesion defects.

Line 758, word missing

Line 772, submitted should be subjected

Line 822, which anti-PilE antibody was used?

Figure 2 should include the pilin structure, showing where the secondary structure elements shown in black map onto it. This is important prior to showing the filament in Figure 3 as those less familiar with the field won't necessarily know how the pilins are organized in the polymer. Right now the pilin structure is not shown until Figure 4.

Referee #3:

General/major

Type IV pili form filament-based assemblies which mediate a variety of functions, notably inter-bacterial aggregation, host cell adhesion, twitching motility and natural competence. The structural and mechanistic basis for mediation of these diverse functions is unclear at present. This manuscript addresses some of these questions in a novel and imaginative way, through the use of deep mutational scanning (DMS) of the major PilE pilin. Although DMS has been used in other areas, this is its first application to the study of type IV pilus function, to my knowledge. The power of such an approach is heavily dependent on the efficiency of the functional screening methods used. Although clearly not perfect (see, for example, the synonymous mutations in Fig 1B), the authors make a convincing case by validating the properties of selected individual PilE mutants and their effects on aggregation, adhesion and pilus length/surface density. Probably the most interesting outcome is the evidence that adhesion to HUVECs is mediated by the pilus tip. The precise manner of host cell adhesion by Neisseria has been a matter of debate for many years- the authors mention the earlier work on the proposed importance of PilC1, for example. Overall, the experiments have been carefully executed and the results are an important contribution to this field.
1. Lines 137-139: Why do mutations within the N-terminal helix of PilE lead to shorter but greater numbers of pili? Is this related to the balance between assembly and contraction?
2. Lines 161 to 172: Q122E and K140Q would alter the charge on the pilus, but mutations which lead to higher aggregation levels do not, for the most part, seem to be charge-based. Precise calculation of surface electrostatics from protein structures is difficult, although there are software

packages which enable modelling. In practice, the influence of surface electrostatics on protein-protein interactions is also highly dependent on ionic strength.
3. Lines 281-289 The authors should note that protein aggregation is a complex but- at least in vitro- well studied phenomenon (eg Tsiolaki et al Amyloid 2017 3, 143-152). In soluble proteins, aggregation is often driven by 'aggregation hotspots'- in some cases, these involve exposed hydrophobic patches which might explain some of the properties of the mutants in Fig 3D. This would explain how pili can exhibit a high level of sequence diversity but also retain aggregative properties.
4. Did the authors look at competence?

Minor
1. Line 17 I think the authors mean 'However' rather than 'Yet'.
2. The authors should review the correct use of pilus (singular) and pili (plural)- there were several instances in the manuscript where 'pilus', instead of 'pili' should have been used (eg line 19).
3. Another issue of inconsistency is the designation of specific residues in PilE- should it be 'Lys140' or 'K140'? Both are used in the manuscript and one form should be selected.
4. Lines 48 to 50 This sentence is badly worded and should be rewritten.
5. Line 324 'involved'.
6. Line 335 Is this study really Systems Biology?
7. Line 543 The relevant statistical tests used should be named.

1st Revision - authors' response 11th Jul 2019

As suggested by the editor, comments concerning other types of pili have been added to the discussion (lines 318-345).

Referee #1:

For the manuscript by Kennouche et al. the authors generate a library of *N. meningitidis* point mutants covering the length of the major pilin, PilE. The library was analyzed for pilus production, pilus-mediated aggregation and adhesion to HUVEC using a clever approach that isolated pilus-producing bacteria (by FACS), aggregation-competent bacteria (by filtering) and adherent cells (by binding) and then subjected both the isolated functional mutants and the input mutants to next generation sequencing and compared the frequency of the mutants in these populations to identify residues involved in each of these three functions. Interesting mutants were generated de novo and analyzed further. They report residues in the N-terminus of the pilin that, when substituted, result in short but more abundant pili, residues around a protruding and exposed lysine on the pilus surface that are involved in aggregation, and, most interestingly, residues at the tip of the pilin that appear to mediate adhesion. The approach is sophisticated and powerful, the analysis is rigorous and the data are beautifully represented. I have only minor concerns, listed below.

The statement in the abstract "Here, we simultaneously determined the regions of PilE involved in pili display, auto-aggregation and adhesion by using deep mutational scanning and mining this extensive functional map." This is somewhat overstated. Specific residues involved in these processes were identified but the results are not exhaustive. Additional regions involved in these processes might have been missed as single amino acid changes in these regions may not disrupt function.

To tone down this statement we simply suggest to remove the word “the” in the sentence to indicate that other regions might not have been taken into account for the reasons indicated by this reviewer. “Here, we simultaneously determined the regions of PilE involved in pili display, auto-aggregation and adhesion by using deep mutational scanning…”

N-terminal mutants: The authors show that the N-terminal mutants do not aggregate and state that deletion of the retraction ATPase "was sufficient to increase piliation of individual bacteria and restore aggregation in mutants with short pili" but they only report aggregation for one of the N-terminal/pilT double mutants and show no pilus images. What does "increase piliation" mean? Longer pili? Because they report that the mutants are already more piliated than WT.

Results describing the 3 mutants of interest and their corresponding *pilT* double mutants are now included in figure 2E. Representative images of the piliation of these different strains are also indicated in figure EV2E.

The data in Fig. EV2E showing adhesion for the N-terminal mutants are important as they show that the short pili are functional, yet they are not cited until much later in the paper (line 203-205).

Reference to these results is now included in the initial description of these mutants (EV2F).

Lys140 region mutants: Mutations encoding 3 basic residues, K103, K144 and H149, disrupt aggregation but not piliation, and E99 mutants increase aggregation. What about K140? Was it not selected as piliated in the library screen because the antibody, 20D9, does not bind well to K140 mutants? E99 mutants are mentioned here as increasing aggregation but no results are shown for de novo E99 mutants. Was this phenotype not borne out?

K140 mutants were not identified in the screen because, as suggested by this reviewer, the 20D9 antibody does not recognize these mutants and they would thus be placed "inappropriately" in the non-piliated category. We initially focused on mutants with decreased phenotypes and mutants with predicted high aggregation in amino acid E99 (E99A, E99Q) were not generated *de novo*.

Discussion: The results showing the involvement of tip-associated residues in adhesion is intriguing. However the Discussion does not adequately address the data in the literature supporting the presence of minor pilins at the pilus tip, which would block any interactions between the major pilin tips and host cell receptors. Could the tip-labeled pili represent broken pili that have their major pilins exposed, whereas the pili bound to cells are bound via their intact minor pilins? This is a controversial and poorly understood aspect of pilus biology that must be more carefully addressed.

The discussion has been modified to have a more inclusive balanced view regarding the pilus tip composition, in particular in light of results in other bacteria (lines 318-345).

The argument that tip-labeled pili (such as the ones shown in in Fig. 5F) could be broken pili can indeed not be excluded and is an interesting hypothesis. Yet, we believe that the inhibition data shown in Fig. 5G rather support a direct role for PilE at the tip and the view that PilE is the protein binding to the cell.

There are several places where the authors refer to supplementary materials that were not made available: "mutagenic primer listed in Supplementary file 1", "Supplementary Table 1", "Supplementary Table 2", "Video 1", "supplementary information".

All files mentioned are now available. We apologize for this issue in the initial submission. The supplementary information file provides in particular the physical principles that determine how tip bound purified pili behave in the presence of flow.

Line 110-112. "... the mutation scores corresponding to auto-aggregation were normalized by the mutation scores corresponding to piliation". I don't understand this scoring. The Y-axis reports a ratio of aggregation/piliation, which should not result in negative numbers. In addition, the data should be skewed to mutants that are piliated but do not aggregate (<1), with few mutants that are not piliated but aggregate (>1).

The sentence has been changed as well as the figure legend to clarify this point. The goal here is to look at aggregation relative to piliation levels. The value in the Y axis is the $\log_2$ of the ratio between the frequency of each single point mutation in the aggregation library relative to the frequency of the same mutation in the piliation library. In other words, a negative value means that the mutation induces a decrease in aggregation that is stronger than the corresponding decrease in piliation.

Line 130-131. Show the Western blot in addition to the band density plot.

An image of a representative western blot is now added in EV 2D.

Line 163-164. Something is missing here - commas? parentheses?

This part has been reworded and simplified.

Line 168-172: Provide more explanation regarding these analyses including whether pili were sheared from the cells and purified.

This information is now included in the main text.

Line 730, 744. "Dotted" should be "solid".

Corrected

Line 759. "Purple" should be "blue".

Dots in figure 4B are purple

The reference formatting is inconsistent in the bibliography.

Reference format is now consistent.

Fig. 3C. What is all the background for the 20D09 Ab binding to K140Q?

For the K140Q mutant we have pushed the thresholding of the images on purpose to show that there is no labeling at all in order to convince the reader that it is not because of a faded labelling that pili are not visible. Background in these immunofluorescences is not higher, we have just made the noise visible. We now mention this difference in thresholding in the figure legends.

Referee #2:

This interesting manuscript describes well-executed and beautifully illustrated studies aimed at distinguishing residues of the Neisseria meningitidis major pilin protein, PilE, that are critical for particular emergent functions of its type IV pilus filaments. These questions are challenging to address due to the multifunctional nature of the system. The authors used saturating mutagenesis of PilE followed by phenotypic assays and deep sequencing to delineate which aspects of the pilin contribute to each trait. They specifically queried adhesion to human cells, aggregation (pilus-pilus interactions), and piliation - an indirect measure of assembly/disassembly capacity.

I remain unconvinced that pili are adhering through a single PilE tip-exposed epitope alone rather than through a combination of this epitope and minor components. Single mutants of pilV or pilX still make pili with the other gene product.

We have to agree with this reviewer that this is both a central point and an open point of discussion. The discussion section has been extensively modified to include alternative scenarios involving other components such as PilV and PilX minor pilins (lines 318-345).

The full discussion of the role of PilX and PilV is perhaps beyond the scope of this manuscript and could be an interesting topic for a minireview or forum type of publication. To respond to this reviewers' comment nonetheless, perhaps a relevant question to ask oneself is: what is the evidence that the PilV and PilX minor pilins play a direct role in adhesion by inserting in the pilus fiber? To our knowledge two main lines of evidence are available but our efforts over the years to understand pilus functions has led us to question this evidence.

1) ***pilV* and *pilX* mutants retain some level of piliation while losing specific type IV pili associated functions.**

Regarding this point our previously published results (Imhaus et al 2014) as well as the current study clearly establish that while these mutants still have pili they have a lot less. Other studies also show this: (i) Winther-Larsen et al in Mol Micro (2005) in *N. gonorrhoeae* with PilL, the equivalent of PilX, and (ii) Sophie Hélaine et al show also in Mol. Micro in 2005 by ELISA that the pilX mutant in *N. meningitidis* express 0.6 times less pili than the WT. The FACS-based approach presented here confirm that piliated bacteria have less pili and, this is new, the proportion of piliated bacteria is also lower (Figure 2D). One could consider that this quantitative change is not so important but we have shown in our previous study (Imhaus et al 2014) using an inducible system and confirmed in the current study that piliation level are tightly linked to function (Figure 1C). The decreased amount of piliation of these mutants is thus sufficient to explain their phenotypes.

2) PilV and PilX co-purify with pili.

Pili purifications are very crude and the western blots used to detected potential interactors are very sensitive. As a consequence many proteins "co-purify" with pili and conclusions from these experiments should be drawn with caution. Appropriate controls are difficult to find for these experiments.

Some commentary from an evolutionary perspective on the fact that most substitutions had little effect on the phenotypes of interest is warranted in the light of the statement on line 59-60 that there is an unusual number of constraints. Does this result imply that the pilin is able to tolerate significant sequence variation at non-critical positions while maintaining function? How do they converge on optimal sequences that maximize multiple functions? When phase variation occurs, are the critical residues maintained?

As this reviewer we were surprised of the number of mutations that either had not effect on pilus expression and function or even enhanced these properties. A comment was added in the first paragraph of the result section to point out the robustness of the pilus system. One could imagine that occurrence of antigenic variation is likely to optimize a resilient solution compatible with frequent sequence changes. This second point was not included in the text as we felt this required a study on its own that went beyond the scope of the current manuscript focused on type IV pili mediated adhesion and aggregation.

Other comments:

Line 35, carried should be mediated

Corrected.

Line 64, Neisseria pili also bind to abiotic surfaces, as shown by the adherence assays using purified pili. How would those interactions differ from those with host cell surfaces?

Neisseria pili have a strong preference for human cellular surfaces. Encapsulated *Neisseria meningitidis* rarely, if ever, bind to plastic or glass surfaces or even mouse cells for instance. The common view is that pili interact with specific human cellular receptors. This likely reflects the lifestyle of *Neisseria spp*. which only live in association with human mucosal surfaces. The reported observations of pili on glass slides were rare events that probably do not reflect interactions normally taking place during *Neisseria spp* lifestyle. On the rare occasions where we could find pili attached to the glass surface, these were adhering along their length and not by the tip, further suggesting that the initial high affinity binding to human cells would take place at the tip of the pilus.

Line 88, what is the epitope recognized by this mAb? Line 152 says the authors 'suspected' its epitope was around residue 140 but do not say why. What does the nanobody recognize?

Both the 20D9 antibody and the nanobody were generated by an immunization with purified whole pili and the precise epitopes are unknown. During its initial characterization it was

shown that the 20D9 mAb does not recognize a sequence variant with amino acid changes in the D-region (aa 120-154) during (Marceau et al 1995 Mol Micro). Since K140 is located in that variable region it was possible that this mutant would not be recognized.

Line 103-4, what proportion of synonymous mutations yielded positive phenotypes?

Looking more carefully at the distribution of synonymous mutations they rather appear to follow a normal distribution centered around 0 as would be expected. The text was modified accordingly.

Line 127, why would one expect a correlation? By piliation, do the authors mean number of pili or the length of those pili?

We did not mean to imply that a correlation between the level of piliation per bacterium and the percentage of piliated bacteria was expected. We are currently unsure of the meaning or mechanism of this intriguing experimental observation. The sentence starts by the word "interestingly" to point out that this is a new observation that will require further studies.

These results were obtained by flow-cytometry analysis of bacterial piliation. The 'level of piliation' reflects the total number of pilin molecules assembled in pili at the surface of the bacteria, regardless of length and number which can only be obtained by microscopy. The sentence was modified to clarify.

Line 149-50, how conserved is this feature among Neisseria? What about other species that have pili that form bundles eg. Vibrio or E. coli?

As indicated in figure 6 the region surrounding amino acid 141 is variable due to antigenic variation. The fact that amino acids responsible for auto-aggregation change following antigenic variation is expected as different variants have different abilities to auto-aggregate (Marceau et al. Mol Micro 1995). This arrangement is therefore not expected to be conserved between species. Yet, the importance of electrostatic interactions has been reported for other species (see Kirn et al. *Mol. MIcrobiol*, 2000 for *V. cholerae*)

Line 169, presumably this was binary (yes/no) and not quantitative?

In the *Neisseria* field, association of a given protein to pili is frequently assessed by purifying pili by shearing in basic conditions and precipitation by ammonium sulfate followed by protein detection by Western blot. The big difficulty of such an assay is that pili preparations obtained are relatively crude and can be a bit variable between experiments. The goal of the graph representation provided in figure 3E is to present of synthetic view of the 3 independent experiments that were done.

Line 183, pilin, not pilus? The epitope is exposed in pili that are under tension - would that be a factor here?

The sentence was rephrased to clarify. Reference to conformational changes under tension are now indicated.

Line 191, this statement seems contradictory - the authors say that they chose the 30 min timepoint to minimize the contribution of aggregation. Why would adherence defects correlate with aggregation defects if pilus length is key for the latter, unless both parameters rely on the expression of a minimal number of pili?

This sentence was removed.

Line 199-201, if the authors performed flow with bacteria stained with mAb SM1, would they not be able to test the idea that pilus tip numbers per cell was important?

Flow cytometry analysis of pili using the SM1 mAb generated signals that were too low to "count" the number of pilus tips unfortunately. This is expected as only a few tips are

available per bacterium. The competition experiment provided in figure 5G shows the importance of pilus tips for adhesion.

Line 233, the persistence length stated here is for labeled pili. What is the role of the flexible cell surface to which the pili are attached in this experiment?

The experiments we present in Fig.5A-B-C were also performed with labeled pili. It is therefore reasonable to use the persistence length of labeled pili in our model. In addition, as discussed below, the most recent evidence using label-free imaging are supportive of such persistence length.

The second element of the presented physical model (point ii) is the flexible surface of the cell whose elasticity tends to position the pilus in a vertical position unless flow is introduced. In our physical model, this effect is described by the parameter A in the Supplementary Information that was not initially available to reviewers apparently.

Line 241, what is the secondary adhesive component in this scenario, and were there mutants in the authors' collection that could not perform this secondary adhesion step?

At this point we have not identified specific amino acids involved in this second mode of adhesion. This now mentioned in the discussion section.

Line 249-50, the work on antibody-mediated inhibition of Haemophilus T4P binding to the host in should be cited here. PMID:25597921

This citation is now inserted in the discussion section (Novotny et al Mol Micro 2015).

Line 270, in addition to - this is part of assembly, not in addition to assembly

This is now corrected.

Line 292 -302,

- the authors often use pilin and pilus interchangeably throughout, which is not accurate. The mutational analysis was on one pilin protein, and the altered sequence may be exposed only in certain regions of the pilus.

These sentences were modified to ensure correct use of the terms pilin and pilus

- The potential role of minor pilins, which are present in these pili according to the authors own Western data, must be considered.

The possible presence of other minor pilins such as PilV and PilX at the pilus tip is now mentioned.

- The SM1 antibody also binds to stretched pili which have the epitope exposed under tension as may occur during adhesion.

This point is now also mentioned in this part of the discussion.

- There is structural evidence from the Jensen lab, corroborated by genetic data from the Burrows lab, that the minor pilins form a complex that primes assembly and thus becomes part of the pilus tip. In the absence of more direct evidence that Neisseria is different, it would be worth keeping an open mind as to the configuration of the pilus tip.

This paragraph was also modified to keep alternative scenarios open, we cannot exclude a participation of other proteins. The extensive work done in *Pseudomonas* on this topic in particular by the Burrows group is now properly cited.

The groundbreaking structural work done by the Jensen group show a minor pilin complex located at the inner membrane in empty machineries (without pili) but not when pili are present. This as a strong argument that minor pilins play a role in pilus assembly but this is at best indirect evidence of a tip location.

Line 311-317, the pili imaged here are labeled, which could affect their persistence length and behavior. The persistence length of 5 microns for P. aeruginosa pili was likewise estimated from thermal fluctuations of labeled pili so may not be accurate. New work by the Persat lab on unlabeled pili using iSCAT imaging may lead to more accurate estimates.

In their beautiful recent iSCAT imaging of pili Tala *et al* did not make any statement on persistence length of pili. At first glance pili seem straight over several microns, even in absence of contact with surfaces. Specifically, the most deflected pilus in their snapshots shown in the right panel of Fig.2a has the deflected angle of 58 degrees and the length of 7 µm. The persistence length is defined as the length at which the filament is deflected over about 68 degrees on average. (Precisely, the average of cosines of deflected angles is 1/e at the persistence length.) Therefore, the iSCAT images suggest that the persistence length is around 7 µm or even longer. This is in good agreement with a persistent length of several microns reported previously and the value we used in our calculations.

Line 331, what is 'unassisted' flow cytometry?

We meant that quantitative values indicated by flow cytometry are given automatically, without human intervention. This was specified to highlight the improvement from our previous paper (Imhaus et al 2014) in which pili length and number was determined by immunofluorescence labeling followed by microscopic observation and "manual" counting. With the reorganization of the discussion this sentence was removed.

Line 334, this hypothesis could be tested using retraction deficient versions of these mutants in combination with the point mutants of PilE that appear to have adhesion defects.

Although interesting, such a study on role of the PilX and PilV minor pilins is perhaps beyond the scope of this manuscript. Nevertheless, evidence already present in the literature are in favor of a rescue of the *pilX* mutant by a *pilT* mutation. Introducing a *pilT* mutation in a *pilX* mutant thus generating a *pilXpilT* double mutant completely restores the aggregation deficiency of this mutant (Helaine et al Mol Micro 2005). Similarly, the *pilT* mutation restore the inability of the *pilX* mutant to induce intracellular signaling (Brissac et al. I&I 2012).

Line 758, word missing

This is corrected.

Line 772, submitted should be subjected

This is corrected.

Line 822, which anti-PilE antibody was used?

A polyclonal antibody generated by immunization with whole pili preparation was used.This is now indicated. The reference to this antibody is indicated in the M&M section (Morand et al. 2004)

Figure 2 should include the pilin structure, showing where the secondary structure elements shown in black map onto it. This is important prior to showing the filament in Figure 3 as those less familiar with the field won't necessarily know how the pilins are organized in the polymer. Right now the pilin structure is not shown until Figure 4.

The structure of the pili monomer is now included in figure 1.

Referee #3:

General/major

Type IV pili form filament-based assemblies which mediate a variety of functions, notably inter-bacterial aggregation, host cell adhesion, twitching motility and natural competence. The structural and mechanistic basis for mediation of these diverse functions is unclear at present. This manuscript addresses some of these questions in a novel and imaginative way, through the use of deep mutational scanning (DMS) of the major PilE pilin. Although DMS has been used in other areas, this is its first application to the study of type IV pilus function, to my knowledge. The power of such an approach is heavily dependent on the efficiency of the functional screening methods used. Although clearly not perfect (see, for example, the synonymous mutations in Fig 1B), the authors make a convincing case by validating the properties of selected individual PilE mutants and their effects on aggregation, adhesion and pilus length/surface density. Probably the most interesting outcome is the evidence that adhesion to HUVECs is mediated by the pilus tip. The precise manner of host cell adhesion by Neisseria has been a matter of debate for many years- the authors mention the earlier work on the proposed importance of PilC1, for example. Overall, the experiments have been carefully executed and the results are an important contribution to this field.

1. Lines 137-139: Why do mutations within the N-terminal helix of PilE lead to shorter but greater numbers of pili? Is this related to the balance between assembly and contraction?

The exact cause for this phenotype is not clear at this stage and this point is briefly explored in the discussion section. Having numerous pili implies that initiation of piliation is more efficient. Having shorter pili implies that the elongation/retraction balance is changed. One could also be the consequence of the other. Having more initiation and more pili could lead to having shorter pili because of a limitation of some component such as the major pilin for instance. A sentence has been added in the discussion to clarify.

2. Lines 161 to 172: Q122E and K140Q would alter the charge on the pilus, but mutations which lead to higher aggregation levels do not, for the most part, seem to be charge-based. Precise calculation of surface electrostatics from protein structures is difficult, although there are software packages which enable modelling. In practice, the influence of surface electrostatics on protein-protein interactions is also highly dependent on ionic strength.

The reviewer is correct. The increased aggregation phenotype could be explained by a local increase in hydrophobicity and we now mention this in the manuscript. Concerning the role of charge in aggregation, we do not favor a hypothesis where it is a global charge change that would affect pilus-pilus interaction. We rather favor a scenario where specific charged amino acid interact with each other.

3. Lines 281-289 The authors should note that protein aggregation is a complex but- at least in vitro- well studied phenomenon (eg Tsiolaki et al Amyloid 2017 3, 143-152). In soluble proteins, aggregation is often driven by 'aggregation hotspots'- in some cases, these involve exposed hydrophobic patches which might explain some of the properties of the mutants in Fig 3D. This would explain how pili can exhibit a high level of sequence diversity but also retain aggregative properties.

This interesting point is now mentioned.

4. Did the authors look at competence?

We did some experiments on competence but did not follow them up to focus on adhesion and aggregation which are closely tied together in the context of interaction with host cells. Also, competence and pilin sequence was previously explored by Obergfell PLOS genetics 2016.

Minor
1. Line 17 I think the authors mean 'However' rather than 'Yet'.
2. The authors should review the correct use of pilus (singular) and pili (plural)- there were several instances in the manuscript where 'pilus', instead of 'pili' should have been used (eg line 19).
3. Another issue of inconsistency is the designation of specific residues in PilE- should it be 'Lys140' or 'K140'? Both are used in the manuscript and one form should be selected.
4. Lines 48 to 50 This sentence is badly worded and should be rewritten.
5. Line 324 'involved'.
6. Line 335 Is this study really Systems Biology?
7. Line 543 The relevant statistical tests used should be named.

All these points have been corrected.

2nd Editorial Decision 6th Aug 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees, who find that their main concerns have been addressed and are now in favour of publication of the manuscript. There remain only a few editorial issues that have to be dealt with before I can extend formal acceptance of the manuscript.

REFEREE REPORTS:

Referee #1:

I am satisfied with the revisions.

Referee #2:

This revised version has addressed my concerns and provides a more balanced discussion of the findings. The title doesn't really reflect the main thrust of the paper, which is to delineate residues involved in different aspects of pilus biology.

2nd Revision - authors' response 10th Sep 2019

The authors performed the requested changes.

3rd Editorial Decision 12th Sep 2019

Thank you very much for providing the final requested changes in the manuscript. I am now pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal. Congratulations on a nice study!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Guillaume Duménil
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2019-102145

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All results in this study are in vitro experimental procedures which have been used routinely in the laboratory and allowed numerous repeats.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	There are no animal studies in this work.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All quantitative values included in this work have been obtained through automated analysis: Flow cytometry, image analysis was performed automated procedures and western blots were quantified.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	A list of the statistical tests used in the study are indicated in the manuscript.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Test were used in appropriate conditions
Is there an estimate of variation within each group of data?	Standard deviations are indicated for all statistical groups.
Is the variance similar between the groups that are being statistically compared?	yes

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used for Western blots and immunofluorescence: (i) polyclonal serum anti-PilE (Morand et al., 2004), anti-PilV (Mikaty et al., 2009) and anti-Rmp4 (Morand, Tattevin et al., 2001); (ii) mouse monoclonal antibody anti-PilE, clone 20D9 (Pujol et al., 1999) and clone SM1 (Virji, Heckels et al., 1983); (iii) Camelidae nanobody anti-PilE clone F10 (Charles-Orszag et al., 2018). The following goat secondary antibodies were used for immunofluorescence, Western blot and flow cytometry: anti-mouse or anti-rabbit IgG (H+L) coupled to horseradish peroxidase (Jackson Immuno-Research Laboratories) and anti-rabbit or anti-mouse IgG (H+L) coupled to Alexa Fluor 488, 568 or 647 (Life Technologies) and mouse anti-His tag (Biolegend). 40,6-diamidino-2-phenylindole (DAPI) was purchased from Life Technologies and Hoechst 33342 from Invitrogen. Trypsin-EDTA (0.05%) was purchased from Gibco.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines in this study were routinely tested for mycoplasma contamination. Huvecs primary endothelial cells were used. They were bought from Promocell.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All the data generated in provided in two tables provided with the manuscript.
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