THE EMBO JOURNAL

In situ and high-resolution cryo-EM structure of a bacterial Type VI secretion system membrane complex

Chiara Rapisarda, Yassine Cherrak, Romain Kooger, Victoria Schmidt, Riccardo Pellarin, Laureen Logger, Eric Cascales, Martin Pilhofer, Eric Durand, Rémi Fronzes

Review timeline:

Submission date:	10th Oct 2018
Editorial Decision:	11th Jan 2019
Revision received:	22nd Jan 2019
Editorial Decision:	1st Feb 2019
Revision received:	4th Feb 2019
Accepted:	6th Feb 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 11th Jan 2019

Thank you for submitting your manuscript for consideration by the EMBO Journal. I sincerely apologise for the unusual delay in the assessment of your work due to belated submission of referee reports. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, the reviewers appreciate the work and the quality of the data. However, they also raise several issues that would need to be addressed before they can support publication here. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees.

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://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. 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, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

REFEREE COMMENTS

Referee #1:

Summary:
Rapisarda et. al present the cryo-electron tomography and single particle cryo-electron microscopy of the T6SS membrane complex from enteroaggregative E. coli. T6SS are contractile machines that mediate the transfer of toxins to both competitor bacterial and eukaryotic cells. They are composed of a membrane complex (MC) stably anchored in the bacterial envelope that propels an Hcp tube and a VgrG spike aiming to puncture the prey cell wall and deliver lytic toxins. The work presented shows an in situ and high-resolution view of the MC following the previous reported negative stain structure published by Durant et al 2015. The structure provides novel insights into the molecular organization of the MC where 5 additional copies of TssJ were identified yielding a revised overall TssJ-L-M stoichiometry of 3:2:2. Mutational analysis and fluorescence localization imaging were used to confirm the interactions seen in the structure for the TssJ' additional subunits and periplasmic gate. Together, this study broadens our knowledge about T6SS architecture and expands our understanding of toxin secretion via the T6S apparatus. The manuscript is clearly written and the data are well presented.

Major points:
Although I do not have any concerns about the C5 symmetry of the complex, I would like to see further validation for the newly described 3:2:2 stoichiometry by SEC-MALS or native mass-spectrometry.
I have two major concerns in relation to the cryo-EM reconstruction of the complex base (Fig EV4D, EV4E and F): i. If the authors would like to claim that the inner membrane sub-domain of the T6SS MC is filled with PE phospholipids than they would have to verify the presence of such lipids in the complex by lipidomics or similar method. ii. The FSC curve calculated shows typical artefacts of tight masking, i.e the FSC curve does not drop to zero. The increase at high frequencies indicates the correlation between masks imposed on the two half maps.

Minor points:
Line 50: correct "(no title)"
Line 143: "The angular distribution of the particles was good..." The sentence should clarify why this particular angular distribution allowed reaching high resolution.
Line 157: The authors describe that the channel is closed by the gate at the intersection between arches and pillars (in the C5 structure). It would be interesting to show that this is also observed in the C1 structure?
Line 762: Reference is incorrect.
Fig. EV1: "A" panel is missing in the figure legend.
Fig. EV2A: Top right box with n values should be described in the legend.
Fig. EV3H: The weaker density for one of the archers is not clear in the figure. The authors should considers a better illustration for this observation.

Referee #2:

In this report, Dr. Fronzes and co-authors have extended their previous work on the T6SS TssJLM membrane complex from a negative stain EM structure to <I>in situ</I> electron cryotomography imaging and high-resolution cryo-EM single particle analysis. This new study has mainly revealed (i) the correct locations of where the bacterial membranes intersect the complex, (ii) a special 3:2:2 stoichiometry among the three protein components TssJ/L/M, (iii) the structure of TssM N-terminal fragment missing in the previous crystal structure, and (iv) a gate in the periplasmic channel as well as its precise residue composition and interactions. Based on the new high-resolution structural information, the authors preformed point-mutagenesis assays aiming to disrupt the newly discovered periplasmic gate or the third TssJ (the TssJ') to test their roles in T6SS function. Overall this is a nice incremental work on the structural understanding of this important molecular machine. However, there are major concerns need to be fully addressed to support some of the key

conclusions.

Major concerns:

Line 222: "these results demonstrate that TssJ'-TssJ.i interface is required for the stability of the T6SS MC, sheath formation and T6SS antibacterial activity." - Based on the images and data provided in the current manuscript, it is unclear whether the TssJ R31 and D97 residues are uniquely important in the TssJ'-TssJ.i interaction but not involved in TssJ.o and/or TssJ.i's association with the complex, or even the structural stability of TssJ. The authors need to provide better information/evidences to support this critical conclusion.

Line 268: "The TssM periplasmic gate is required for MC assembly and <I>T6SS function</I>" - Although it is highly likely that the TssM periplasmic gate is tightly associated with proper T6SS function, the point-mutated TssM variants generated in this study all have disrupted the assembly of the membrane complex. Therefore, it is still inconclusive that whether a constitutively close or open gate in a properly assembled T6SS machine will affect the function.

Other issues:

Line 50: "...(No Title)"?

Line 64: "...T4SSb" - please use the full name when first introduce this abbreviation

Line 233: I cannot tell where in the Fig.5A are the 8 helices mentioned.

Line 246: Base on the image shown in Fig. EV9F. It is very unconvincing to say that the poorly-defined density is a helix and belongs to TssM.

There are several "TssJ.a" throughout the main text and figure legend. Please correct them.

The "external/internal" and "outer/inner" pillar seem to be used interchangeably in the manuscript. I suggest to just pick one to prevent confusion.

Referee #3:

The Type VI secretion system (T6SS) is a molecular injection apparatus that is used by Gram-negative bacteria to translocate effectors across their inner and outer membranes into contacting prokaryotic and eukaryotic cells. In this process a nanotube, consisting of stacked Hcp hexamers, tipped with an arrowhead-shaped protein (VgrG) punctures the target cell and toxic effectors are delivered either into the cytoplasm or periplasm. For anchorage of the T6SS apparatus within the donor cell, a trans-envelope complex is required.

Recently, a low resolution negative stain electron microscopy structure of the membrane-spanning complex (MC) from enteroaggregative Escherichia coli (EAEC), consisting of TssJ, TssM and TssL, was reported, exhibiting a 5-fold rotational symmetry with two centric rings of pillars and arches that spans the periplasm.

In the current manuscript by Rapisarda et al, the authors confirm the 5-fold symmetry of the MC in situ by cryo-electron tomography and present a high-resolution model of purified MC obtained by single particle cryo-EM. The in situ structure revealed the orientation of the entire complex within the donor cell and allowed the identification of membrane-inserted regions. The overall architecture of the MC, elucidated by single particle cryo-EM, showed the presence of five additional copies of TssJ, resulting in a revised total stoichiometry of 3:2:2 for TssJ:TssL:TssM (2:2:2 was proposed based on the negative staining structure). Mutational studies in combination with in vivo assays support an essential role of this third copy of TssJ in MC assembly and T6SS activity. Furthermore, a periplasmic gate formed by a 11-residue loop of TssM was identified, that was shown to be crucial - similar as the additional TssJ - for MC assembly and T6SS function. Altogether, the authors propose a refined model of the mechanism of action of the MC during assembly and function of the T6SS.

This is an exciting manuscript that provides not only the so far elusive in situ data of the MC, but also novel insights into the organization of the complex on a molecular level. Although important parts of the complex, like the base/cytosolic domains or the transmembrane helices could not be resolved, the insights into periplasm-spanning channel significantly advances our understanding of this crucial part of the T6SS.
There are a few minor things that should be addressed in more detail in the discussion such as the basal part that are visible in some of the cryotomographic slices (Fig. 2C) or the role of the transmembrane regions of the individual components. Also the fact that the loop region that builds the periplasmic gate is less conserved and its putative implications could be discussed in more detail.
Additional cryo-ET experiments with the mutants would further strengthen the claim that the MC assembly/tail polymerization is compromised in the mutant strains. However, this can also be done in a follow-up study.
The model presented in Fig. 6 is rather speculative and needs to be toned down. Experimental findings backing-up the model should be specifically provided for each panel in the legend.

Specific comments:

1. In Fig. 4E: I cannot see a significant difference in the fluorescence images between WT and the D97K cells as indicated in l. 212-213. The data on time-lapse recordings are more convincing, presented in EV7A, B. One can consider exchanging these results.
2. Fig. 4 legend: Labeling of subunits in the legend vs. figure/text is inconsistent: TssJ.a should be TssJ.i, TssJ.A should be TssJ.o. Same applies to TssM.
3. Fig. 4D: "Prey cells (Gfp+ kanR E. coli W3110) were mixed with the indicated attacker 432 cells, spotted onto Sci-1-inducing medium (SIM) agar plates and incubated for 4 h at 37{degree sign}C" might go to M & M part. See also Fig. 5E
4. Fig. 5A: Rotation arrows should be shown or alternatively top and bottom view labeled.
5. l. 331. TssJ' interacts with TssJ,o (not TssJ,I, as it is written)
6. l. 332 same as in 5.

1st Revision - authors' response 22nd Jan 2019

Itemized response to referees' comments.

Referee #1:

Summary:
Rapisarda et. al present the cryo-electron tomography and single particle cryo-electron microscopy of the T6SS membrane complex from enteroaggregative E. coli. T6SS are contractile machines that mediate the transfer of toxins to both competitor bacterial and eukaryotic cells. They are composed of a membrane complex (MC) stably anchored in the bacterial envelope that propels an Hcp tube and a VgrG spike aiming to puncture the prey cell wall and deliver lytic toxins. The work presented shows an in situ and high-resolution view of the MC following the previous reported negative stain structure published by Durant et al 2015. The structure provides novel insights into the molecular organization of the MC where 5 additional copies of TssJ were identified yielding a revised overall TssJ-L-M stoichiometry of 3:2:2. Mutational analysis and fluorescence localization imaging were used to confirm the interactions seen in the structure for the TssJ' additional subunits and periplasmic gate. Together, this study broadens our knowledge about T6SS architecture and expands our understanding of toxin secretion via the T6S apparatus. The manuscript is clearly written and the data are well presented.

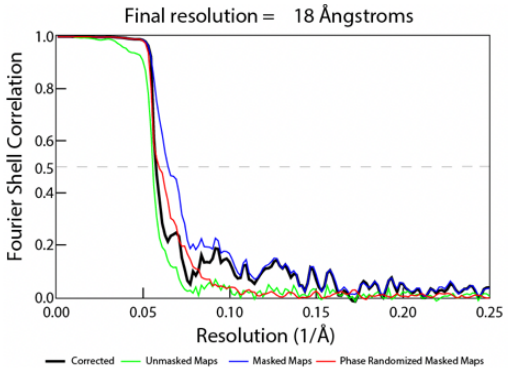
Major points:
Although I do not have any concerns about the C5 symmetry of the complex, I would like to see further validation for the newly described 3:2:2 stoichiometry by SEC-MALS or native mass-spectrometry.
Biochemical validation of this stoichiometry is tricky. Indeed, the purified TssJLM complex is highly unstable and the purified material quite heterogenous. We had to collect a very large cryoEM dataset

to identify a sufficient amount of properly assembled complexes to the solve the structure. Such sorting is not possible for biochemical or biophysical methods. All our attempts to further stabilize the complex to obtain a more homogenous sample did not improve its quality. Due to these limitations regarding sample quality, SEC-MALLS or native mass spectrometry would not help in obtaining reliable information concerning the stoichiometry of the complex.
Labelling methods could also have been used, but again the heterogenous nature of the sample prevented us in obtaining reliable data concerning the stoichiometry.

I have two major concerns in relation to the cryo-EM reconstruction of the complex base (Fig EV4D, EV4E and F): i. If the authors would like to claim that the inner membrane sub-domain of the T6SS MC is filled with PE phospholipids than they would have to verify the presence of such lipids in the complex by lipidomics or similar method.
The TssJLM membrane complex is embedded in two bacterial membranes. We use a detergent mix to extract the complexes from the membranes and maintain them as soluble as possible. We describe densities that could correspond to a lipid bilayer in both the high-resolution cryoEM density map and the CryoET map. We propose that these densities could be a lipid bilayer but, indeed, we do not have further validation of this hypothesis. Lipidomics or similar approaches will tell us that lipids have been co-purified with the TssJLM complex. We expect that it would be the case since we extracted the protein from membranes. Membrane proteins always co-purify with some lipids from their original environment (as described in the literature. i.e Palsdottir and Hunte, BBA, 2004). However, unfortunately lipidomics or biochemical approaches would not tell us where are these lipids in the density map.
That being say, we agree with the reviewer that we need to tone down our claim that the T6SS inner membrane complex is filled with a lipid bilayer.
We modified the text accordingly: "*We propose that the inner membrane sub-domain of the T6SS MC could be filled by a lipid bilayer. However, such hypothesis will have to be further explored in the future.*"

ii. The FSC curve calculated shows typical artefacts of tight masking, i.e the FSC curve does not drop to zero. The increase at high frequencies indicates the correlation between masks imposed on the two half maps.
We have corrected the FSC curve using a mask that was extended by 10 pixels instead of 5 and the line drops to 0.

F



Minor points:
Line 50: correct "(no title)"
Corrected the (no title) to the Nature Microbiology paper that was not yet accepted for publication when this paper was submitted.

Line 143: "The angular distribution of the particles was good..." The sentence should clarify why this particular angular distribution allowed reaching high resolution.
We removed this sentence because it is misleading. The angular distribution of the particles is not the only key element that allowed 3D reconstruction at high-resolution. This comment is not necessary in the main text.

Line 157: The authors describe that the channel is closed by the gate at the intersection between arches and pillars (in the C5 structure). It would be interesting to show that this is also observed in the C1 structure?

We have added *Appendix Fig S2H*, the corresponding figure of the full complex slices (Fig3B-E) for the C1 complex and referenced to it in the main text.
We added the following sentence: "*This gate is also visible in the reconstruction without symmetry applied (Appendix Fig S2H)"*

Line 762: Reference is incorrect.
We have added the relevant information for the reference of line 762 and all the others that were missing issue and page numbers.

Fig. EV1: "A" panel is missing in the figure legend.
We have added the "A" in the figure legend.

Fig. EV2A: Top right box with n values should be described in the legend.
We edited the text as follows and changed the Figure EV2 to Figure S1:
"**A** FSC curve of the C5 symmetrized final average. The *n* value in the top right box corresponds to the number of particles in each of the two equal-sized groups whose averages are being compared in the Fourier space."

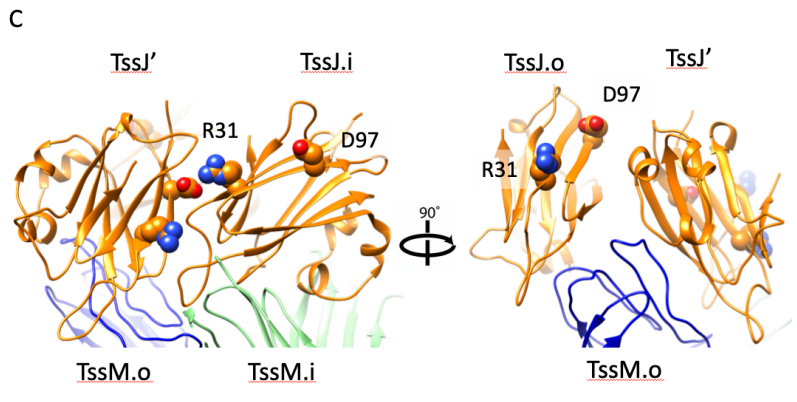
Fig. EV3H: The weaker density for one of the archers is not clear in the figure. The authors should considers a better illustration for this observation.
We have changed the angle at which we took the picture to make it clearer that one arch is less well-defined than the others. We hope that the new figures are clearer. See *Appendix Fig S2H*

Referee #2:

In this report, Dr. Fronzes and co-authors have extended their previous work on the T6SS TssJLM membrane complex from a negative stain EM structure to *in situ* electron cryotomography imaging and high-resolution cryo-EM single particle analysis. This new study has mainly revealed (i) the correct locations of where the bacterial membranes intersect the complex, (ii) a special 3:2:2 stoichiometry among the three protein components TssJ/L/M, (iii) the structure of TssM N-terminal fragment missing in the previous crystal structure, and (iv) a gate in the periplasmic channel as well as its precise residue composition and interactions. Based on the new high-resolution structural information, the authors preformed point-mutagenesis assays aiming to disrupt the newly discovered periplasmic gate or the third TssJ (the TssJ') to test their roles in T6SS function. Overall this is a nice incremental work on the structural understanding of this important molecular machine. However, there are major concerns need to be fully addressed to support some of the key conclusions.

Major concerns:

Line 222: "these results demonstrate that TssJ'-TssJ.i interface is required for the stability of the T6SS MC, sheath formation and T6SS antibacterial activity." - Based on the images and data provided in the current manuscript, it is unclear whether the TssJ R31 and D97 residues are uniquely important in the TssJ'-TssJ.i interaction but not involved in TssJ.o and/or TssJ.i's association with the complex, or even the structural stability of TssJ. The authors need to provide better information/evidences to support this critical conclusion.
We have made a new figure (now EV Figure 3). We hope that it shows that the two residues R31 and D97 are not involved in any bonds, either with TssM or with other TssJ that are not between TssJ.o and TssJ'. The interaction between TssJs is absent or very weak and we have discussed the bonds and the energy required to break them apart in the Table 1 calculated with the PISA software



Line 268: "The TssM periplasmic gate is required for MC assembly and *T6SS function*" - Although it is highly likely that the TssM periplasmic gate is tightly associated with proper T6SS function, the point-mutated TssM variants generated in this study all have disrupted the assembly of the membrane complex. Therefore, it is still inconclusive that whether a constitutively close or open gate in a properly assembled T6SS machine will affect the function.
We fully agree with the reviewer. We changed the title of the paragraph to: "*The TssM periplasmic gate is required for MC assembly*"

Other issues:

Line 50: "...(No Title)"?
See above – The no title was used a place marker for the paper on the baseplate that had not been published yet. We have fixed the reference accordingly.

Line 64: "...T4SSb" - please use the full name when first introduce this abbreviation
We modified the text as suggested

Line 233: I cannot tell where in the Fig.5A are the 8 helices mentioned.
We have added an extra panel in Figure 5 (B) where the 8 helices are more clearly visible.

Line 246: Base on the image shown in Fig. EV9F. It is very unconvincing to say that the poorly-defined density is a helix and belongs to TssM.

We have changed the text to indicate that the loop was built mainly to show that the remaining amino acids are not sufficient to reach the membrane as it was previously postulated in Durand et al 2015.
Note that EV9F was renumbered to EV10F
The new text is now: "*A poorly-defined density that sits in the core region between TssM.i and TssM.o^{+1}, was attributed to the C-terminus of TssM. If we were to build a small loop that terminates into a helix at the C-terminus, we would not be able to reach the membrane region as previously proposed (Durand et al, 2015) (Fig EV4F). This same loop is disordered in the outer pillar monomer (TssM.o).*"

There are several "TssJ.a" throughout the main text and figure legend. Please correct them.
We have corrected all of the incorrectly labelled TssJs and TssMs (as we were previously naming them)

The "external/internal" and "outer/inner" pillar seem to be used interchangeably in the manuscript. I suggest to just pick one to prevent confusion.
We agree with the referee that using only one terminology is the best way to prevent confusion for the reader. We have chosen to use outer/inner and have changed all the instances where external/internal appeared.

Referee #3:

The Type VI secretion system (T6SS) is a molecular injection apparatus that is used by Gram-negative bacteria to translocate effectors across their inner and outer membranes into contacting prokaryotic and eukaryotic cells. In this process a nanotube, consisting of stacked Hcp hexamers, tipped with an arrowhead-shaped protein (VgrG) punctures the target cell and toxic effectors are delivered either into the cytoplasm or periplasm. For anchorage of the T6SS apparatus within the donor cell, a trans-envelope complex is required.

Recently, a low resolution negative stain electron microscopy structure of the membrane-spanning complex (MC) from enteroaggregative Escherichia coli (EAEC), consisting of TssJ, TssM and TssL, was reported, exhibiting a 5-fold rotational symmetry with two centric rings of pillars and arches that spans the periplasm.

In the current manuscript by Rapisarda et al, the authors confirm the 5-fold symmetry of the MC in situ by cryo-electron tomography and present a high-resolution model of purified MC obtained by single particle cryo-EM. The in situ structure revealed the orientation of the entire complex within the donor cell and allowed the identification of membrane-inserted regions. The overall architecture of the MC, elucidated by single particle cryo-EM, showed the presence of five additional copies of TssJ, resulting in a revised total stoichiometry of 3:2:2 for TssJ:TssL:TssM (2:2:2 was proposed based on the negative staining structure). Mutational studies in combination with in vivo assays support an essential role of this third copy of TssJ in MC assembly and T6SS activity. Furthermore, a periplasmic gate formed by a 11-residue loop of TssM was identified, that was shown to be crucial - similar as the additional TssJ - for MC assembly and T6SS function. Altogether, the authors propose a refined model of the mechanism of action of the MC during assembly and function of the T6SS.

This is an exciting manuscript that provides not only the so far elusive in situ data of the MC, but also novel insights into the organization of the complex on a molecular level. Although important parts of the complex, like the base/cytosolic domains or the transmembrane helices could not be resolved, the insights into periplasm-spanning channel significantly advances our understanding of this crucial part of the T6SS.
There are a few minor things that should be addressed in more detail in the discussion such as the basal part that are visible in some of the cryotomographic slices (Fig. 2C) or the role of the transmembrane regions of the individual components. Also the fact that the loop region that builds the periplasmic gate is less conserved and its putative implications could be discussed in more detail.

We have added the following sentence in the discussion.
"*These cytoplasmic densities, which we can assume would be connected to the baseplate in a fully assembled T6SS, had a heterogeneous appearance that highlighted the flexibility of the base, as discussed in the next paragraph*."

Additional cryo-ET experiments with the mutants would further strengthen the claim that the MC assembly/tail polymerization is compromised in the mutant strains. However, this can also be done in a follow-up study.
We fully agree with the reviewer.

The model presented in Fig. 6 is rather speculative and needs to be toned down. Experimental findings backing-up the model should be specifically provided for each panel in the legend.
We agree that this model is speculative. We modified the figure 6 accordingly and we removed the state N°3 for which we have very little evidence (complex "priming"). The other states were already proposed in other studies (Durand et al. 2015, Zoued et al. 2016) and review articles in the literature. We also added the following sentences in the text: " *This model is still uncomplete and rather speculative. Many aspects of the secretion mechanism by the T6SS remain elusive. Further investigations are therefore needed to provide a complete molecular understanding of this mechanism."*

Specific comments:

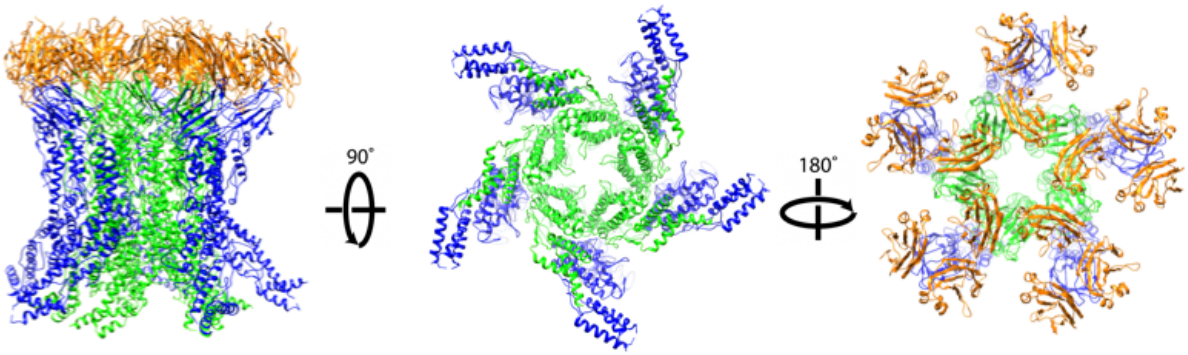
1. In Fig. 4E: I cannot see a significant difference in the fluorescence images between WT and the D97K cells as indicated in l. 212-213. The data on time-lapse recordings are more convincing, presented in EV7A, B. One can consider exchanging these results.

We have exchanged the panels as the reviewer suggested.

2. Fig. 4 legend: Labeling of subunits in the legend vs. figure/text is inconsistent: TssJ.a should be TssJ.i, TssJ.A should be TssJ.o. Same applies to TssM.
We have corrected all of the incorrectly labelled TssJ and TssMs (as we were previously naming them)

3. Fig. 4D: "Prey cells (Gfp+ kanR E. coli W3110) were mixed with the indicated attacker 432 cells, spotted onto Sci-1-inducing medium (SIM) agar plates and incubated for 4 h at 37{degree sign}C" might go to M & M part. See also Fig. 5E
We have changed the legend of both figures (Fig 4D and Fig 5E) to not reiterate the details in Materials and methods.

4. Fig. 5A: Rotation arrows should be shown or alternatively top and bottom view labeled.
As suggested by the reviewer we have added rotation arrows to better show the views of the model.
We have also added rotation arrows where they were missing in the rest of the figures.



5. l. 331. TssJ' interacts with TssJ,o (not TssJ,I, as it is written)
We have modified the text accordingly

6. l. 332 same as in 5.
See above

Additional changes:

To fit the formatting required by the EMBO journal, we have chosen 4 expanded view figures and have moved all the others ones to the Appendix file (included in the submission).
To help the reviewers and the editors better assess the changes we have made, we created a table where the old numbering is compared side to side with the new one.
The main Figures are unchanged.

Old table number	**New Figure number**
EV1	Table 1
EV2	Appendix Table S1
EV3	Appendix Table S2
EV4	Table 2
EV5	Table 3
Old Figure number	**New Figure number**
EV1	EV1
EV2	Appendix Figure S1
EV3	Appendix Figure S2
EV4	Appendix Figure S4
EV5	EV2
EV6	EV3
EV7	Appendix Figure S5
EV8	Appendix Figure S6
EV9	EV4
EV10	Appendix Figure S7
EV11	Appendix Figure S8
EV12	Appendix Figure S9
EV13	Appendix Figure S10
EV14	Appendix Figure S11
EV15	Appendix Figure S12

2nd Editorial Decision 1st Feb 2019

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by all referees, who find that their main concerns have been addressed and now recommend publication of the article. There remain only a few minor editorial issues that have to be resolved before formal acceptance of the manuscript.

REFEREE REPORTS:

Referee #1:

The authors have addressed all my concerns and the revised manuscript is now ready for publication. Overall,
the findings are very exciting and they provide novel insights into the structure of the Type VI secretion membrane complex.

Referee #2:

The authors have addressed all concerns raised by this referee.

Referee #3:

All points have been addressed. The manuscript is ready for publication.

2nd Revision - authors' response 4th Feb 2019

All requested editorial changes were made.

3rd Editorial Decision 6th Feb 2019

Thank you for incorporating the final changes into 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:	Rémi Fronzes
Journal Submitted to:	EMBO journal
Manuscript Number:	EMBOJ-2018-100886R

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?	No statistical methods were used to determine sample sizes. However, sample sizes indicated in figure legend were such that standard error of the mean were within a confidence interval of 99 %. Similar sample sizes have been already used for equivalent studies reported in Durand et al., (2015) and Zoued et al., (2016).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not Applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analysis
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 the experiments were performed with a random selection of microscopy fields, cells and clones. All experiments were performed with clonal populations.
For animal studies, include a statement about randomization even if no randomization was used.	Not Applicable
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.	No blinding was performed as the acquisition and analysis methods require human intervention.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not Applicable
5. For every figure, are statistical tests justified as appropriate?	All attempts at replication were successful. Experiments were done in triplicate, each with three independent biological samples. Statistical analyses are provided, or a representative experiments is shown. All information are indicated in the Methods section, legend and corresponding figures
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Floating bars representing the number of detected foci for each strain were made using GraphPad (https://www.graphpad.com). Microscopy analyses were performed at least three times, each in technical triplicate, and a representative experiment is shown.
Is there an estimate of variation within each group of data?	Not Applicable
Is the variance similar between the groups that are being statistically compared?	Not Applicable

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	Commercial antibodies: -Mouse monoclonal anti-VSVG, clone P5D4 (Sigma-Aldrich, Cat# A5977, Lot# 018M4841V, dilution 1/2000) -Mouse monoclonal anti-FLAG, clone M2 (Sigma-Aldrich, Cat# F3165, Lot# SLBN8915V, dilution 1/2000) -Mouse monoclonal anti-HA, clone HA-7 (Sigma-Aldrich, Cat# H3663, Lot# 066M4837, dilution 1/2000) -Mouse monoclonal anti-StrepII, clone GT661 (Iba, Cat# 2-1507-001, Lot# 41246, dilution 1/500) -Mouse monoclonal anti-polyHistidine, clone AD1.1.10 (Bio-Rad, Cat# MCA1396GA, Lot# 1701, dilution 1/1000) -Alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Millipore, Cat# AP503A, Lot# 134002, dilution 1/2000) All antibodies were validated in western-blots with samples expressing tagged and un-tagged protein.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable

* 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.	Not Applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not Applicable
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.	Not Applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not Applicable
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.	Not Applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not Applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not Applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable
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.	Not Applicable
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.	Not Applicable

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	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable