THE EMBO JOURNAL

Manuscript EMBO-2017-97515

# Hierarchical protein targeting and secretion is controlled by an affinity switch in the type III secretion system of enteropathogenic Escherichia coli

Athina G. Portaliou, Konstantinos C. Tsolis, Maria S. Loos, Vassileia Balabanidou, Josep Rayo, Alexandra Tsirigotaki, Valerie F. Crepin, Gad Frankel, Charalampos G. Kalodimos, Spyridoula Karamanou & Anastassios Economou

*Corresponding authors: Spyridoula Karamanou and Anastassios Economou, KU Leuven*

**Review timeline:**

| | |
|---|---|
| Submission date: | 06 June 2017 |
| Editorial Decision: | 17 July 2017 |
| Revision received: | 26 July 2017 |
| Editorial Decision: | 01 September 2017 |
| Revision received: | 05 September 2017 |
| Accepted: | 11 October 2017 |

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 17 July 2017

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

As you can see from the comments, all reviewers appreciate the significance of the presented mechanism of T3SS assembly. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript while addressing the comments of all reviewers, but particularly focusing on further characterisation of the inner membrane vesicle system, as requested by referees #1 and #2.

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, we request that you contact the editor as soon as possible upon publication of any related work to discuss how to proceed.

 

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

---
REFEREE REPORTS

Referee #1:

Pathogenic bacteria utilize type III secretion systems (T3SSs) to inject effector proteins into their host cells, thereby causing a wide variety of infectious diseases. The type III protein export apparatus ensures the order of protein targeting and secretion. A transmembrane protein of the T3SS of EPEC, EscV, provides binding-sites for type III chaperone/substrate complexes to coordinate protein secretion in a strict hierarchical manner. SepL and SepD are required for the switching of export specificity of the export apparatus from middle (translocators) to late export substrates (effectors) upon contact with host cells but it remains unknown how they do. In the present study, the authors have developed in vitro reconstitution of T3S substrate targeting and hierarchical switching using inverted membrane vesicles (IMVs) and showed that

1. An unfolded C-terminal tail of the CesAB chaperone specific for EspA is required for membrane targeting of the CesAB/EspA complex;
2. The CesAB/EspA complex requires SepL and EscV for efficient targeting to the IMVs of EPEC;
3. SepL binds to the membrane through its N-terminal segment;
4. Both SepL and EscV are required for strong binding of the CesAB/EspA complex to the IMVs;
5. An interaction between SepL and CesAB is required for efficient targeting of the CesAB/EspA complex to the IMV and the C-terminal tail of CesAB is required for this interaction;
6. SepD regulates the SepL- and EscV-C-dependent membrane targeting of the CesAB/EspA complex;
7. SepD and SepL regulate the binding affinities of EscV for CesAB/EspA and CesT/Tir chaperone-substrate complexes;
8. These interactions are required for the strict order of protein secretion, allowing EPEC cells to infect HeLa cells.

These results led to a plausible hypothesis that physical communications among SepL, SepD and EscV may establish the strict order of protein targeting and secretion. Overall, the paper is reasonably well organized and clearly written. The methods seem reliable, and the results and conclusion are sound scientifically. This research article would be of great interest to general readership, providing important advancements in our knowledge on the hierarchical type III protein targeting and secretion. However, this reviewer has several comments and the following comments would hopefully help improve the manuscript.

Comments:
1. It has been shown that flagellar type III chaperone-substrate complexes bind to the C-terminal cytoplasmic domain of FlhA (FlhA-C) for efficient unfolding and translocation of the substrates by the export apparatus and that relatively well-conserved Asp456, Phe459 and Thr490, which are located at an interface between domains D1 and D2 of FlhA-C, are involved in the interaction with highly conserved Tyr residues of flagellar chaperones. It has been also shown that export substrates increase the binding affinity of their cognate chaperones for FlhA-C and that different binding affinities of FlhA-C for these chaperone-substrate complex confers an advantage for the strict order of flagellar filament assembly. Therefore, the authors should cite the following papers and discuss these observations in the Discussion section.

(1) Bange, G. et al. FlhA provides the adaptor for coordinated delivery of late flagella building blocks to the type III secretion system. Proc. Natl. Acad. Sci. USA 107, 11295-11300 (2010).
(2) Minamino, T. et al. Interaction of a bacterial flagellar chaperone FlgN with FlhA is required for efficient export of its cognate substrates. Mol. Microbiol. 83, 775-788 (2012).
(3) Kinoshita, M. et al. Interactions of bacterial chaperone-substrate complexes with FlhA contribute to co-ordinating assembly of the flagellar filament. Mol. Microbiol. 90,1249-1261 (2013).

 

The following two paper describes that the unfolded C-terminal tail of FlgN chaperone, which contains a highly conserved Tyr-122 residue is responsible for the interaction with FlhA-C.

(1) Minamino, T. et al. Interaction of a bacterial flagellar chaperone FlgN with FlhA is required for efficient export of its cognate substrates. Mol. Microbiol. 83, 775-788 (2012).
(2) Kinoshita, M. et al. Rearrangements of α-helical structures of FlgN chaperone control the binding affinity for its cognate substrates during flagellar type III export. Mol. Microbiol. 101, 656-670 (2016).

The following paper describe that FliS chaperone promote FliC docking at the FlhA-C platform to facilitate subsequent unfolding and translocation of FliC.

(1) Furukawa, Y. et al. Structural stability of flagellin subunit affects the rate of flagellin export in the absence of FliS chaperone. Mol. Microbiol. 102, 405-416 (2016).

2. Figures 2C, 3B, 3C, 3D, 3F, 4C, 4F, 4H, 6: The authors should carry out statistical analysis to show statistically significant differences.

3. Fig. S2C: They showed that EscV is in the membrane vesicle and that the C-terminal cytoplasmic domain is present outside the membrane vesicle as judged by immuno-gold electron microscopy. However, it remains unclear that EscV is located inside the inner ring complex of the injectisome of EPEC because this reviewer did not see the inner ring structure. Therefore, to clarify it, the author should carry out co-localization analysis. Samuel Wagner and his co-workers have reported that the assembly of InvA into the type III export apparatus is required for SpaP, SpaQ and SpaR. Therefore, the authors should prepare inverted membrane vesicles from EPEC mutant strains lacking the SpaP, SpaQ and SpaR homologues and carry out immuno-gold electron microscopic analysis. In addition, they should use these mutant strains as negative control for flotation assays to clarify that the EscV ring structure is the primary binding sites for chaperone-substrate complexes.

4. P8, line 152-153: The CesAB/EspA complex bound to the IMVs prepared from EPEC but not those from BL31. But, this result did not tell that the EPEC vesicle contains the injectisome. Therefore, the authors should tone down their statement if they cannot show that the injectisome exits inside the IMVs.

5. Chapter "CesAB/EspA targeting to the injectisome requires SepL and EscV: Deletions of EscN and EscQ significantly reduced the levels of SepL targeted to the membranes. But, neither of deletions affected the binding of the CesAB/EspA complex to the IMVs. Why? Please provide a clear answer. A truncation of the C-terminal domain of EscU reduced not only the level of the CesAB/EspA complex to the membrane vesicles by about 70% of the wild-type level but also the SepL level, raising the possibility that the C-terminal domain of EscU also contributes to the membrane targeting of the CesAB/EspA complex. Evans et al. have shown that the C-terminal cytoplasmic domain of FlhB, which is a flagellar homologue of EscV, provides for export substrates (Evans et al. 2013. Nature 504, 877-882). Therefore, this reviewer thinks that the authors should focus on EscV in addition to SepL and EscV. What do you think about that?

6. Chapter "SepL physically interacts with the C-domain of EscV: As mentioned above, depletions of EscN, EscQ and the C-domain of EscU considerably reduced the levels of membrane-localized SepL as well (Fig. 3). So, there is the possibility that they are required for proper and stable anchoring of SepL to the membrane.

7. P11, lines 207-208: How did the author identify the γ site is located on the distinct protomer? Peptide arrays did not tell you that SepL binds to the α and β sites on one protomer and the γ site on its neighboring protomer in the nonameric ring model of EscV. So, the authors should change to "SepL binds to three distinct, α, β and γ sites of the C-domain of EscV. Since MixA-C and its homologue FlhA-C have four compactly folded domains, D1, D2, D3 and D4, they also should show which domains contain these binding sites in the Figure.

8. p11, lines 221-223: This reviewer agreed with the authors that EscV-C and SepL contribute to stable association of the CesAB/EspA with the IMVs. However, depletions of EscN and EscQ considerably reduced the levels of membrane-localized SepL but did not affect the membrane

 

localization of the EesAB/EspA complex. This observation may not allow the authors to describe their conclusion.

9. P13, lines 270 - 271: This reviewer thinks that the mutation in the α site of EscV-C has reduced the levels of membrane-associated SepL by 50% of the wild-type level (Fig. F4, row 3). The authors should carry out statistical analysis.

10. P14, lines 282 - 283: As mentioned above, the authors should carry out statistical analysis. Otherwise, the authors cannot mention that a depletion of SepD affects neither membrane association nor receptor function of SepL. Mutations in the β and γ sites of the C-domain of EscV did not affect the membrane association of SepL but significantly reduced the binding affinity of the CesAB/EspA complex for the IMVs. However, the depletion of SepD allows the CesAB/EspA complex to bind to these EscV-C mutant vesicles. Since SepL binds to three distinct, α, β and γ sites of EscV-C, there is the possibility that these mutations increase the binding affinity for SepD and decreases that for SepL. As a result, the CesAB/EspA complex cannot bind to a physical interface between EscV-C and SepL.

Referee #2:

This is a very comprehensive study of bacterial type III secretion system in enteropathogenic E. coli. The manuscript is well written. The results and figures are nicely presented. The major concern is the isolation of inverted inner membrane vesicles (IMVs). As the most critical step of the methods, more details and validation are needed to establish the model system. More importantly, immune-gold electron microscopy was only used to confirm EscV on the surface of IMVs. Is it possible to show co-localization of the ATPase EscN and EscV in the IMVs. The cytoplasmic complexes including the C ring and the ATPase are highly delicate, therefore it may take additional steps to confirm the presence and integrity of the complexes after purification of IMVs.

Referee #3:

The leading labs of Economou/Karamanou, Kalodimos and Frankel have dissected the targeting and hierarchical secretion mechanism of the Type III secretion system using in vitro and in vivo tools and EPEC as a model system. Specifically, they have reconstituted the Type III targeting reaction in vitro using inverted inner membrane vesicles, and use this in conjunction with peptide array and mass spectrometry assays to probe the hierarchy of secretion and quantify affinity constants. Two sets of chaperone/exported protein pairs are used, representing respectively the middle exported substrates (translocators) and late substrates (effectors).

Using these tools, they establish that the translocator pair (CesAB/EspA) is targeted to the membrane, define components of these proteins that act as targeting signals and define SepL and EscV as major membrane binding sites. They go on to determine that the so-called gatekeeper protein of the system, SepL, associates at the membrane on or at the EscV protein, the major component of the export apparatus. In so doing the gatekeeper enhances the affinity for translocator substrates and reduces that of effectors by >15 fold. This effectively decides the secretion hierarchy by promoting the export of translocators and disfavouring that of effectors.

This is a clearly written manuscript, describing rigorous quantitative experimental work that dissects the molecular basis of the targeting hierarchy in Type III secretion and lays the foundations for the complete functional reconstitution of Type III secretion in vitro. This is a remarkable study that presents the first in vitro reconstitution of the very complex Type III apparatus building on the foundations laid by Wickner on the Sec system. Crucially, for the first time protein interactions with membrane components of the Type III secretion apparatus are quantified (i.e. in a physiological setting). The authors have taken a step-wise approach to deal with the complexity of the Type III system and focus on targeting here. They identify new functional targeting elements on CesAB, then proceed to identify the receptor using gene knockouts and peptide arrays. Finally, they complement this robust in vitro analysis with in vivo functional analysis of all the mutants they have generated.

 

One rarely sees such a complete chain of experimentation.

The study presents a significant advance in our understanding, with well-supported conclusions. How the Type III system functions, how its targeting works and how its substrate hierarchy is decided has remained obscure. A main reason for this was the absence of in vitro reconstitution tools. Without these, conclusions were drawn based on static structural snapshots and protein interaction studies carried out either in the experimentally challenging in vivo setting or with purified components in the absence of their physiological partners, and crucially in the absence of membranes. The hierarchy control that is mediated by the SepL gatekeeper is a completely novel role for these proteins that are generally thought of as being themselves exported proteins. SepL is known be important in the switching mechanism since gene knock outs cause the system to switch to effectors and block translocator export but how this switching is achieved at a molecular level was completely unclear. The key finding here is that SepL appears to act as a quasi-secretory substrate itself but once it occupies the export apparatus it prevents effector export and becomes effectively a receptor subunit for translocators! Furthermore, the study reveals that regulation of the affinity switch can be brought about by simply removing the SepD chaperone. Most current models propose that SepL/gatekeeper secretion will be essential to allow switching. Here the authors demonstrate that removal of the chaperone is sufficient.

The work constitutes a major contribution in the field and will be of general interest to researchers studying protein trafficking and protein machines. In my view, this manuscript should be published as is.

Minor corrections
Line 274 "Mutating the SepL-binding interfaces of EscV significantly affected the receptor function of wild-type SepL". Does receptor function mean CesAB/EspA binding, in which case this sentence appears to be redundant given the preceding sentence "Nevertheless, all derivatives lost high affinity CesAB/EspA binding. If it refers to something else, can the authors please clarify?

Line 305 "drastically" misspelled

Line 306 reference to Fig 4A should be to Figure 5A

Line 406 There appears to be a missing word after "inter-dependent"

1st Revision - authors' response 26 July 2017

**Portaliou et al Rebuttal**
We thank the reviewers for excellent comments that help clarify the experiments and their interpretations. Please find below all the points raised, addressed one by one.

**Reviewer #1**
*"Pathogenic bacteria utilize type III secretion systems (T3SSs) to inject effector proteins into their host cells, thereby causing a wide variety of infectious diseases. The type III protein export apparatus ensures the order of protein targeting and secretion. …This research article would be of great interest to general readership, providing important advancements in our knowledge on the hierarchical type III protein targeting and secretion…"*
**Authors' response:**
We thank the reviewer for the kind words and the appreciation of the importance of our study.

**Point #1.**
*Comments:*
*It has been shown that flagellar type III chaperone-substrate complexes bind to the C-terminal cytoplasmic domain of FlhA (FlhA-C) for efficient unfolding and translocation of the substrates by the export apparatus and that relatively well-conserved Asp456, Phe459 and Thr490, which are located at an interface between domains D1 and D2 of FlhA-C, are involved in the interaction with highly conserved Tyr residues of flagellar chaperones. It has been also shown that export substrates increase the binding affinity of their cognate chaperones for FlhA-C and that different binding*

 

*affinities of FlhA-C for these chaperone-substrate complex confers an advantage for the strict order of flagellar filament assembly. Therefore, the authors should cite the following papers and discuss these observations in the Discussion section.*
*(1) Bange, G. et al. ...*
*(2) Minamino, T. et al. ...*
*(3) Kinoshita, M. et al….*
*...*
*(1) Minamino, T. et al….*
*(2) Kinoshita, M. et al. ...*
*...*
*(1) Furukawa, Y. et al. ...*

**Authors' response:**
We thank the reviewer for suggesting expanding our discussion section to include some aspects of the related flagellar mechanism and prominent contributions from Prof. Minamino's group.
**Action taken:** References and flagellar system findings are now elaborated in the discussion.

**Point #2.**
*"Figures 2C, 3B, 3C, 3D, 3F, 4C, 4F, 4H, 6: The authors should carry out statistical analysis to show statistically significant differences."*
**Action taken:** Statistical analysis now included with statistical significance and adjusted p values.

**Point #3.**
*"Fig. S2C: They showed that EscV is in the membrane vesicle and that the C-terminal cytoplasmic domain is present outside the membrane vesicle as judged by immuno-gold electron microscopy. However, it remains unclear that EscV is located inside the inner ring complex of the injectisome of EPEC because this reviewer did not see the inner ring structure.*

**Authors' response:**
The IMVs we analyzed were derived from the wild-type EPEC strain that are secreting T3SS proteins, from EspA fillaments detectable by anti-EspA antibodies and are competent at infecting HeLa cells. IN IMVs derived from these cells we have identified >33 LEE and 5 non-LEE proteins of the ~45 proteins of the T3SS by MS (Table S2). Therefore, there is no doubt that these cells have injectisomes. Cell disruption does not lead to loss of injectisomes as shown in numerous studies in the past and as to be expected for membrane-embedded structures that can only be disrupted by detergents. Inverted inner membrane vesicles, as generated here, have been used in hundreds of studies to monitor transmembrane transport. We confirm here their functionality using as a control the Sec system and we show the presence of injectisome components by MS and western immunoblotting, to confirm the presence of several of the components. As the reviewer correctly points out Prof Wagner's group has done a systematic quantitative MS analysis of similar membranes and here we confirm several components from their findings without any firther need of duplicating their work. The immunoEM characterization of the IMVs was aimed at providing supporting additional evidence to the much more detailed MS analysis and also to confirm the ultrastructural features and dimensions of the IMVs. ImmunoEM is inadequate to study injectisome structure and we never intended using it for this purpose in our study.

*"….Therefore, to clarify it, the author should carry out co-localization analysis. Samuel Wagner and his co-workers have reported that the assembly of InvA into the type III export apparatus is required for SpaP, SpaQ and SpaR. Therefore, the authors should prepare inverted membrane vesicles from EPEC mutant strains lacking the SpaP, SpaQ and SpaR homologues and carry out immuno-gold electron microscopic analysis. In addition, they should use these mutant strains as negative control for flotation assays to clarify that the EscV ring structure is the primary binding sites for chaperone-substrate complexes."*

**Authors' response:**
We have not done any systematic effort to express parts of the injectisome and we have not dissected the order of events and whether the assembly of EscV into the type III export apparatus is required for EscR,S,T. What Wagner et al show is that EscV may be dissociated from the injectisome using various detergent treatments but we do not use any detergents here. We have used wild type IMVs that contain fully assembled injectisomes, probably missing the external needles that are immaterial to our studies. So, there is no evidence to suggest that the injectisome should be anything but assembled. This is validated by demonstration of the presence of the EscV, EscU, EscJ, EscD and EscI proteins on these IMVs by MS and antibodies as representative molecules of a

 

tightly assembled structure that can be purified after harsh detergent and salt treatments in many labs. EscU was identified by one high confidence peptide in the cleavable C-domain but was reduced after urea treatment of the IMVs and therefore is not included in Figure 2C. We have not been able to detect EscRST by MS because our peripheral trypsinolysis of the vesicles does not derive appropriate fragments for MS detection and/or their abundance is low or they are overshadowed by other peptides. More specifically, using in-silico digestion, the following peptides are predicted to be MS-detectable after surface proteolysis:

a. 3 peptides for EscR, from the cytoplasmic loop between the TM2 and TM3 domains (LGNETILK pos. 130-137; DNVEFFER, pos. 117-124; DSLFILLPAFTMGQLEAAFK, pos. 139-158).
b. 0 peptides for EscS
c. 0 peptides for EscT.

We also failed to detect the EscR, S, T components using specific peptide antibodies for these proteins because our antibodies have very weak cross-reactivity even against the peptides used as antigens.

Nevertheless, of all the injectisome proteins our study mainly focuses on the nonameric major export subunit EscV. For this we identify specific sights that will bind SepL and Cest/Tir and which when mutated loose these properties. A potential role of EscRST in this reception process is well beyond the scope of our study.

**Action taken:**

1. Detailed information regarding all the peptides identified, proteins, protein abundance, and other MS analysis-related results, has been included in Table S2 (Supplementary material; Appendix) and information more explcitely stated in the text (lines 139-146).

**Point #4.**

*P8, line 152-153: The CesAB/EspA complex bound to the IMVs prepared from EPEC but not those from BL31. But, this result did not tell that the EPEC vesicle contains the injectisome. Therefore, the authors should tone down their statement if they cannot show that the injectisome exits inside the IMVs.*

**Authors' response:**

With the exception of extracellular needles that seem to commonly break off, the remaining part of the injectisome should be unaffected as discussed above. What the flotation and detailed quantitative titration binding studies reveal is that:

a. CesAB/EspA do not bind non-specifically to E.coli lipids or unrelated proteins present in the BL31 IMVs.

b. Binding of CesAB/EspA occurs specifically to EPEC-derived IMVs derived from cells that contained injectisomes.

c. Binding of chaperones/substrates to EPEC-derived IMVs occurs on a saturable high affinity receptor with a nanoMolar $K_d$.

d. Receptor binding on EPEC-derived IMVs is abolished when the C-domain of EscV is mutated or deleted. Therefore, EscV, ogether with SepL, are a or the core component of the receptor.

We have entertained the possibility of using strains missing the Ler transcription factor but we always get a basal level of expression.

**Action taken:** We have clarified these points better in the text (lines 157-159).

**Point #5.**

*"A) Chapter "CesAB/EspA targeting to the injectisome requires SepL and EscV: Deletions of EscN and EscQ significantly reduced the levels of SepL targeted to the membranes. But, neither of deletions affected the binding of the CesAB/EspA complex to the IMVs. Why? Please provide a clear answer."*

**Authors' response:**

The amount of SepL that is found localized in the membrane is indeed somewhat affected when EscQ, EscN or the C-domain of EscU were deleted. Presumably, these proteins help stabilize large amounts of SepL at the membrane but we do not know the molecular basis for this. It is also clear from our data that the small amounts of SepL detected in the IMVs derived from those strains were sufficient for CesAB/EspA targeting to occur. We presume this is because small amounts of SepL are stoichiometric to EscV and these are enough to act as a receptor and EscV allosteric regulator. However, more SepL molecules may accumulate around the translocase.

 

**Action taken:** We have clarified these points in lines 190-192.

**Point #6**
*"B) A truncation of the C-terminal domain of EscU reduced not only the level of the CesAB/EspA complex to the membrane vesicles by about 70% of the wild-type level but also the SepL level, raising the possibility that the C-terminal domain of EscU also contributes to the membrane targeting of the CesAB/EspA complex. Evans et al. have shown that the C-terminal cytoplasmic domain of FlhB, which is a flagellar homologue of EscU, provides for export substrates (Evans et al. 2013. Nature 504, 877-882). Therefore, this reviewer thinks that the authors should focus on EscU in addition to SepL and EscV. What do you think about that?"*
**Authors' response:**
The C-domain of EscU is indeed somehow important for membrane localization of high amounts of SepL. However, SepL is still found in the membrane in low but measurable amounts and SepL can still act fully as a CesAB receptor. Therefore, while EscU clearly affects SepL localization, this is only a regulatory capacity. This regulatory role of EscU is beyond the scope of our study where we focus on the essential components.
**Action taken:** We have clarified these points in lines 190-192.

**Point #7.**
*"Chapter "SepL physically interacts with the C-domain of EscV: As mentioned above, depletions of EscN, EscQ and the C-domain of EscU considerably reduced the levels of membrane-localized SepL as well (Fig. 3). So, there is the possibility that they are required for proper and stable anchoring of SepL to the membrane."*
**Authors' response:**
High level membrane localization of SepL seems to be affected from the deletions of the EscQ and of the C-domain of EscU. However, as mentioned in **#6** above, these proteins have only regulatory roles that are not central to this stage of our effort.

**Point #8.**
*"P11, lines 207-208: How did the author identify the γ site is located on the distinct protomer? Peptide arrays did not tell you that SepL binds to the α and β sites on one protomer and the γ site on its neighboring protomer in the nonameric ring model of EscV. So, the authors should change to "SepL binds to three distinct, α, β and γ sites of the C-domain of EscV. Since MixA-C and its homologue FlhA-C have four compactly folded domains, D1, D2, D3 and D4, they also should show which domains contain these binding sites in the Figure."*
**Authors' response:**
The reviewer's comment helps us clarify this point. The potential binding sites of SepL identified by peptide array analysis were mapped on a single protomer of EscV and on the nonameric model structure of the EscV C-domain. Sites a and b are proximal on the structure of the same protomer forming a continuous apparent binding surface, but site g is located on the opposite site of the protomer. Therefore, as an alternative visualization model we place it at the adjacent protomer. In this case, the three sites would line the walls of a groove on the EscV nonamer. This hypothetical model awaits additional structural data for testing.
**Action taken**:

1. Mapping of binding sites on the nonamer model better explained (lines 216-221).
2. To avoid over-burdening the structure, we have indicated the 4 domains D1-D4 on the EscV C-domain sequence map of Figure EV4F.

**Point #9.**
*p11, lines 221-223: This reviewer agreed with the authors that EscV-C and SepL contribute to stable association of the CesAB/EspA with the IMVs. However, depletions of EscN and EscQ considerably reduced the levels of membrane-localized SepL but did not affect the membrane localization of the CesAB/EspA complex. This observation may not allow the authors to describe their conclusion."*
**Authors' response:**
Please see response to comments #5 and #6. Despite the loss of SepL material, its CesAB/EspA receptor function, a core focus of this study, remains unaffected.

 

**Point #10.**
*"P13, lines 270 - 271: This reviewer thinks that the mutation in the α site of EscV-C has reduced the levels of membrane-associated SepL by 50% of the wild-type level (Fig. F4, row 3). The authors should carry out statistical analysis."*
**Authors' response:**
SepL amounts detected on IMVs derived from the EscV patch a mutant are compromised compared to the wt one. However, SepL remains stably associated to the membrane, presumably on the other EscV C-domain sites and independently with its N-terminus. The marginally less amount of SepL detected on the EscV patch a mutant is not sufficient to justify the 13-fold loss of CesAB/EspA affinity for those membranes.
**Action taken:**
Statistical analysis now included.

**Point #11.**
*A) P14, lines 282 - 283: As mentioned above, the authors should carry out statistical analysis. Otherwise, the authors cannot mention that a depletion of SepD affects neither membrane association nor receptor function of SepL.*
**Authors' response:**
Although there should be no dispute when changes of orders of magnitude are observed, statistical analysis was included. See also response to comments #5 and #6.

**Point #12**
*B) Mutations in the β and γ sites of the C-domain of EscV did not affect the membrane association of SepL but significantly reduced the binding affinity of the CesAB/EspA complex for the IMVs. However, the depletion of SepD allows the CesAB/EspA complex to bind to these EscV-C mutant vesicles. Since SepL binds to three distinct, α, β and γ sites of EscV-C, there is the possibility that these mutations increase the binding affinity for SepD and decreases that for SepL. As a result, the CesAB/EspA complex cannot bind to a physical interface between EscV-C and SepL.*
**Authors' response:**
We have not looked for independent SepD interaction with EscV as it is quite obvious from structural data available from SepD, CesL and their homologues (O'Connell et al., 2004; Vizcaino et al., 2016; Younis et al., 2010) that this protein has a defined role as a SepL chaperone. Given that in its absence, SepL still goes to the membrane at EscV, SepD need not be necessarily directly involved in interacting with EscV. Moreover, we have not observed SepD membrane localization in the absence of SepL (data not shown). In any case, it would be unusual for loss of function mutations on the predicted EscV-SepL interface to enhance the binding of a factor. Nevertheless, such an interaction can also not be excluded currently.
**Action taken:** We have mentioned this possibility in the discussion (lines 463-465).

**Reviewer #2**
**#1**
*"This is a very comprehensive study of bacterial type III secretion system in enteropathogenic E. coli. The manuscript is well written. The results and figures are nicely presented."*
**Authors' response:**
We thank the reviewer for the kind words and the appreciation of the importance of our study.

**#2**
*"The major concern is the isolation of inverted inner membrane vesicles (IMVs). As the most critical step of the methods, more details and validation are needed to establish the model system. More importantly, immune-gold electron microscopy was only used to confirm EscV on the surface of IMVs. Is it possible to show co-localization of the ATPase EscN and EscV in the IMVs. The cytoplasmic complexes including the C ring and the ATPase are highly delicate, therefore it may take additional steps to confirm the presence and integrity of the complexes after purification of IMVs"*
**Authors' response:**
We sense from both reviewers 1 and 2 that there are concerns about IMVs because these biochemicals are new to the T3S field although they are an established tool in bacterial membrane biochemistry for the past 50 years. The cytoplasmic components (EscN ATPase and EscQ, the main

C-ring component) are indeed not detected in our IMVs preparations (that are competent for binding CesAB/EspA and CesT/Tir), neither with MS (Fig. 2C; Supplementary table 2) nor with immuno-staining (data not shown). Therefore it would also not be possible to detect these two components by immunoEM. These observations and those of others over the years, that failed to detect the peripheral cytoplasmic components on purified injectisomes, makes us suppose that these components are detached from the injectisome during the preparation of the IMVs. Other components of the C-ring that were identified using MS analysis, such as EscL and EscK, may form more stable associations.

**Reviewer #3**

**#1**

*"The leading labs of Economou/Karamanou, Kalodimos and Frankel have dissected the targeting and hierarchical secretion mechanism of the Type III secretion system ……The work constitutes a major contribution in the field and will be of general interest to researchers studying protein trafficking and protein machines. In my view, this manuscript should be published as is."*

**Authors' response:**

We thank the reviewer for the kind words and the appreciation of the importance of our study.

**#2**

*Minor corrections*

**Action taken:**

Done

**References:**

O'Connell, C.B., Creasey, E.A., Knutton, S., Elliott, S., Crowther, L.J., Luo, W., Albert, M.J., Kaper, J.B., Frankel, G., and Donnenberg, M.S. (2004). SepL, a protein required for enteropathogenic Escherichia coli type III translocation, interacts with secretion component SepD. Molecular microbiology *52*, 1613-1625.

Vizcaino, J.A., Csordas, A., del-Toro, N., Dianes, J.A., Griss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T.*, et al.* (2016). 2016 update of the PRIDE database and its related tools. Nucleic acids research *44*, D447-456.

Younis, R., Bingle, L.E., Rollauer, S., Munera, D., Busby, S.J., Johnson, S., Deane, J.E., Lea, S.M., Frankel, G., and Pallen, M.J. (2010). SepL resembles an aberrant effector in binding to a class 1 type III secretion chaperone and carrying an N-terminal secretion signal. Journal of bacteriology *192*, 6093-6098.

2nd Editorial Decision 01 September 2017

Thank you for submitting a revised version of your manuscript. I apologise for the delay in communicating the decision due to delayed referee reports. The manuscript has now been seen by two of the original referees, who find that their main concerns have been addressed. There remain some editorial issues that have to be resolved before I can accept the manuscript for publication.

Please let me know if you have any further questions regarding this final revision. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to receiving the final version.

---

REFEREE REPORTS

Referee #1:

Thank you very much for your great efforts to make an revision based on all reviewers' comments and suggestions. The authors' response to my concerns now make this reviewer clear. and This reviewer very much feels that the revised MS is now suitable for publication in EMBO J.

Referee #2:

The revision has addressed my previous concerns.

2nd Revision - authors' response 5 September 2017

Authors made requested editorial changes.

3rd Editorial Decision 11 October 2017

Thank you again for your patience during the final revision process. I am now pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Congratulations!

 

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: Anastassios Economou, Spyridoula Karamanou |
| Journal Submitted to: EMBO J |
| Manuscript Number: EMBOJ-2017-97515 |

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

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 χ2 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.**

USEFUL LINKS FOR COMPLETING THIS FORM

| | |
|---|---|
| http://www.antibodypedia.com | Antibodypedia |
| http://1degreebio.org | 1DegreeBio |
| http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo | ARRIVE Guidelines |
| http://grants.nih.gov/grants/olaw/olaw.htm | NIH Guidelines in animal use |
| http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm | MRC Guidelines on animal use |
| http://ClinicalTrials.gov | Clinical Trial registration |
| http://www.consort-statement.org | CONSORT Flow Diagram |
| http://www.consort-statement.org/checklists/view/32-consort/66-title | CONSORT Check List |
| http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum | REMARK Reporting Guidelines (marker prognostic studies) |
| http://datadryad.org | Dryad |
| http://figshare.com | Figshare |
| http://www.ncbi.nlm.nih.gov/gap | dbGAP |
| http://www.ebi.ac.uk/ega | EGA |
| http://biomodels.net/ | Biomodels Database |
| http://biomodels.net/miriam/ | MIRIAM Guidelines |
| http://jjj.biochem.sun.ac.za | JWS Online |
| http://oba.od.nih.gov/biosecurity/biosecurity_documents.html | Biosecurity Documents from NIH |
| http://www.selectagents.gov/ | List of Select Agents |

**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? | n/a |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | n/a |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | n/a |
| 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. | n/a |
| For animal studies, include a statement about randomization even if no randomization was used. | n/a |
| 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. | n/a |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | n/a |
| 5. For every figure, are statistical tests justified as appropriate? | yes |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | n/a |
| Is there an estimate of variation within each group of data? | yes |
| Is the variance similar between the groups that are being statistically compared? | yes |

**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). | done |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | n/a |

* 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. | n/a |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | n/a |
| 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. | n/a |

**E- Human Subjects**

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | n/a |
| 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. | n/a |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | n/a |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | n/a |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | n/a |
| 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. | n/a |
| 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. | n/a |

**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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | Source data includes: 1 excel file with complete list of proteins /peptides identified by LC- MS/MS that have been submitted to the Pride repository.<br>MS data are available via ProteomeXchange with identifier PXD007087. |
| 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). | MS data deposited to Pride |
| 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). | n/a |
| 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. | n/a |

**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. | no |