Chimeric adaptor proteins translocate diverse type VI secretion system effectors in Vibrio cholerae

Daniel Unterweger, Ben Kostiuk, Rina Oetjengerdes, Ashley Wilton, Laura Diaz-Satizabal and Stefan Pukatzki

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Transaction Report:

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

Editor: David del Álamo

1st Editorial Decision 09 March 2015

Thank you for the submission of your manuscript entitled "Chimeric adaptor proteins translocate diverse type VI secretion system effectors in Vibrio cholerae". We have now received the reports from the referees, which I copy below.

As you can see from their comments, all three referees are very supportive of the publication of your study in The EMBO Journal, but point out to a number of concerns, that will require your attention before we can accept your manuscript. In particular, besides minor comments and some technical issues, both referees #1 and #3 agree that the final part of the paper, regarding the recombination experiments with Tap-1, must be further strengthen and suggest several experiments along this line. I think these suggestions are fairly clear and reasonable but please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

In light of this assessments, I would naturally like to invite you to submit a revised version of your work. We normally allow a single round of major revision only, which should be submitted within the next three months. Should you foresee a problem in meeting the three-month deadline, please let us know in advance and we may be able to grant an extension.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, I would appreciate if you contact me as soon as possible upon publication of any related work in order to discuss how to proceed.
When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://emboj.msubmit.net/html/emboj_author_instructions.html - a2.12

As you have probably seen already, every paper now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Finally, in order to ensure good reporting standards and to improve the reproducibility of published results, our guidelines to authors are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Accordingly, we now require the submission of a completed author checklist, which covers in a systematic manner your practices regarding animal welfare, human subjects, data deposition, statistics and research ethics. It needs to be filled (not all fields may apply to your study in particular) and returned to the editorial office at revision, either via the online submission system as a supplementary file or by email (contact@embojournal.org). Please, click on the link below and follow the instructions to download the checklist file:

http://emboj.embopress.org/authorguide

Again, please contact me at any time during revision if you need any help or have further questions.

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

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REFEREE REPORTS

Referee #1:

The study by Unterweger et al. provides an important insight into mechanism of secretion of T6SS substrates and is of an interest for a broad T6SS field. Authors identified an adaptor protein named Tap-1 that binds to VgrG and its cognate effector protein and is essential for the secretion. Bioinformatic analysis shows that Tap-1 proteins are wide spread in many bacterial species, suggesting a conserved general mechanism. Tap-1 proteins cluster into three distinct families that differ in sequence but also share certain conserved sequence motives. Furthermore, authors suggested a potential mechanism for generation of novel effector combinations through recombination in tap-1, which is however experimentally the weakest part of the paper. All bioinformatic analyses are convincing and very interesting. Tap-1 was clearly shown to be important for secretion of TseL. Authors also clearly demonstrated the importance of Tap-1 structure for secretion of cognate effectors in various strains of V. cholerae. The study would be much stronger if the following major concerns were addressed.

Major concerns:
1) Even though VgrG-1 involvement in secretion of TseL was suggested by the killing experiments, this is complicated by the fact that VgrG-1 deletion also decreases Hcp secretion (as properly acknowledged by the authors). This part of the study (Figure 3) is a bit weak and not completely convincing. One possible way to resolve this issue would be to generate a VgrG-1 mutant that secretes Hcp at wt level but abolishes TseL secretion and then to test if this correlates with abolished biding of Tap-1 to VgrG-1. Alternatively, authors could try to complement VgrG-1 deletion in V52 strain by expression of VgrG-1 from 1587 strain (or another strain that is predicted to have VrgG-1 incompatible with Tap-1 of V52 strain). Again, abolished secretion of TseL should correlate with abolished binding of VgrG-1 to Tap-1. Such analysis would significantly strengthen this part of the study and the paper overall.
2) Experiments showing interactions between Tap-1, VgrG-1 and TseL are well performed and convincing, however, it is not clear if the identified interactions are direct or if additional proteins are involved, such as VgrG-3 (as shown by Dong et al. PNAS 2013). This part of the study would be
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much stronger if the authors tested the interaction experiments in E. coli. The previous observations made by Dong et al. PNAS 2013 should be discussed in the paper.

3) An experiment that tests for presence of Tap-1 in the supernatant should be part of the main text and at least a supplementary figure should be provided to show the results.

4) Even though the possible recombination in tap-1 gene is intriguing, the experimental evidence for this is the weakest part of the study and should be either removed or substantially improved. The recombination experiments are poorly described and lack appropriate controls. The paper is interesting and novel enough even without the recombination experiment. If however the authors want to keep this part of the study, more experiments elucidating the mechanism of recombination should be added. Some experiments to consider are for example:

- measurement of recombination efficiency
- dependency on the highly conserved and homologous region of tap-1 as well as other parts of tap-1 and cognate effector genes
- dependency on recA
- test if this is universal for any DNA sequences around the conserved site in tap-1 gene
- Does it depend on other Vibrio genes, or would this happen in E. coli as well?

Minor concerns:

1) Lines 294-298: It is not completely clear which Tap-1 sequences were analyzed. Figures 5A, C show that class 1 and 2 Tap-1 proteins have very similar N-termini, but class 3 is different (shorter). Were the class 3 Tap-1 proteins part of the alignment in Figure E6 or not? If yes, then how did you deal with the difference in length of class 3 Tap-1 proteins? Please clarify this part of the text and also Figure legends.

2) Line 297: 61% identity, figure E6 shows 64%

Referee #2:

The study by Unterweger et al. identified a new adaptor protein, Tap-1, which is required for proper loading of type VI secretion system (T6SS) effector proteins onto the "weapon tip" of the T6SS tube (e.g., VgrG-associated). The authors used a bioinformatics approach to elucidate the diversity of this protein with respect to it's N-terminal and C-terminal domain and how the diversity mimics the diversity of the up-and down-stream encoded VgrGs and effector proteins, respectively. Indeed, the manuscript convincingly shows that the Tap-1 protein is a chimeric protein. Next, the authors used genetic and biochemical approaches to show that Tap-1 interacts with VgrG-1 and also the effector protein TseL. Moreover, using immunoprecipitation the authors were able to show that Tap-1 links the VgrG-1 protein and the effector protein TseL and that Tap-1 is specific to VgrG-1 (and not universal for other VgrG proteins that are present in V. cholerae).

In summary, this study is of great importance to better understand the loading of the T6SS system but also to better understand the evolution of V. cholerae strains and how genes encoding tap-1, effector and immunity proteins might be co-transferred between strains to change the predator's killing / competition potential.

The manuscript is extremely well written and very sound (in fact, I very much enjoyed studying the details of the manuscript). The experiments are well performed and contain all required controls. Altogether this publication will be a great asset to the field and beyond.

There are no major points of critique. Thus, we solely provide minor comments below, which aim at further improving the quality and clarity of the manuscript.

Minor concerns that should be addressed

• Fig. 1: as the authors refer in the text and experiment later on to vasW and vasX it would be good to indicate these genes in Fig. 1C (in addition to the VCA0019 label).

• Fig. 3: the authors mention in the text that vgrG-1 deletion leads to less Hcp secretion (as can be seen in the figure). How come this phenotype cannot be complemented with the vgrG-1-carrying plasmid in trans? Does the mutant have a polar effect (e.g., is transcription of the downstream genes affected)? Doesn't seem to be the case though on the TseL levels in the cell pellet)?
The authors would like to conclude that these results indicate that recombination occurred between the C6706 tap1 allele (the allele on the chromosome) and tap1 on the plasmid. They sequenced the PCR products and found that the 5’ end of the tap1 allele contained on the plasmid and that it occurred within the 99 bp terminal segments vary with the sequence of the effector proteins encoded downstream of tap1. Additional sequence comparisons suggest that highly conserved sequences within tap1 homologs in a variety of V. cholerae strains, the authors find that Tap1 proteins appear to be chimeric and the sequences of their C-terminal segments vary with the sequence of the effector proteins encoded downstream of tap1. The authors then show that, indeed, the C-terminus of Tap1 is required for translocation of the specific effector encoded downstream of tap1. Additional sequence comparisons suggest that highly conserved sequences within tap1 may be involved in recombination and the authors present an experiment to test this hypothesis. According to the manuscript, the authors transformed strain C6706 with a plasmid containing tap1 and its downstream effector- and immunity-encoding genes from strain 1587, passaged the transformant several times, and then performed PCR using a forward primer that anneals to the 5’ end of tap1 on the chromosome and a reverse primer that is specific for the 3’ end of the tap1 allele contained on the plasmid. They sequenced the PCR products and found that the 5’ half (up to the 99 bp of identity between the alleles) of the PCR product corresponded to the C6706 tap1 gene and the 3’ half corresponded to the 1587 tap1 allele (the allele on the plasmid). The authors would like to conclude that these results indicate that recombination occurred between tap1 on the chromosome and tap1 on the plasmid and that it occurred within the 99 bp terminal segments.
conserved region. However, the experiment lacks the important control of demonstrating that the result is not simply a PCR artifact (that is, that they would not obtain the same result even if recombination had not occurred). Moreover, details of the experimental design are lacking. For example, what DNA exactly is contained on the plasmid - specifically, are sequences 3' to the immunity-encoding gene present? If the chimeric PCR product that the authors obtained is not an artifact, did it result from a single recombination event that occurred at the 99 bp region? Or did a double recombination event occur such that tap-1 plus the effector- and immunity-encoding genes were transferred to the chromosome? It seems that a better experimental design could have been used to determine not only if recombination within the 99 bp can occur, but that it does occur and that the resulting strain with its newly acquired tap-1-effector-immunity module has a competitive advantage or an ability to coexist with other strains, as suggested in the abstract.

Minor concerns:
1. Line 29: What do the authors mean by "In our model"?
2. Many statements throughout the introduction require references. Examples include the sentences ending on lines 46, 51, 52, 57, 74, 86, 88, . . .
3. Line 139: "the" should be "they"
4. Line 338: "encoding" should be "containing"
5. Lines 359-360: What is the evidence supporting this statement?
6. Lines 505-508: This experiment must be explained in much more detail.

1st Revision - authors' response 07 June 2015

Point-by-point response to the referees’ comments

Referee #1:
The study by Unterweger et al. provides an important insight into mechanism of secretion of T6SS substrates and is of an interest for a broad T6SS field. Authors identified an adaptor protein named Tap-1 that binds to VgrG and its cognate effector protein and is essential for the secretion. Bioinformatic analysis shows that Tap-1 proteins are wide spread in many bacterial species, suggesting a conserved general mechanism. Tap-1 proteins cluster into three distinct families that differ in sequence but also share certain conserved sequence motives. Furthermore, authors suggested a potential mechanism for generation of novel effector combinations through recombination in tap-1, which is however experimentally the weakest part of the paper. All bioinformatic analyses are convincing and very interesting. Tap-1 was clearly shown to be important for secretion of TseL. Authors also clearly demonstrated the importance of Tap-1 structure for secretion of cognate effectors in various strains of V. cholerae. The study would be much stronger if the following major concerns were addressed.

Major concerns:
1) Even though VgrG-1 involvement in secretion of TseL was suggested by the killing experiments, this is complicated by the fact that VgrG-1 deletion also decreases Hcp secretion (as properly acknowledged by the authors). This part of the study (Figure 3) is a bit weak and not completely convincing. One possible way to resolve this issue would be to generate a VgrG-1 mutant that secretes Hcp at wt level but abolishes TseL secretion and then to test if this correlates with abolished binding of Tap-1 to VgrG-1. Alternatively, authors could try to complement VgrG-1 deletion in V52 strain by expression of VgrG-1 from 1587 strain (or another strain that is predicted to have VrgG-1 incompatible with Tap-1 of V52 strain). Again, abolished secretion of TseL should correlate with abolished binding of VgrG-1 to Tap-1. Such analysis would significantly strengthen this part of the study and the paper overall.

The reviewer suggests two experiments: (1) to generate a VgrG-1 mutant that
secretes Hcp at wild-type levels, but abolishes TseL secretion, in order to test if this correlates with abolished binding of Tap-1 to VgrG-1, or (2) to complement a vgrG-1 deletion in strain V52 by expression of VgrG-1 from strain 1587 (or another incompatible strain). While preparing for experiment 2, we created a chimeric Tap-1 version containing the N-terminal portion from 1587, the conserved core, and the C-terminal portion from V52. This chimeric Tap-1 was able to complement a tap-1 null-mutation in V52, suggesting that VgrG1 from 1587 and V52 share a conserved Tap-1-binding region. Thus, it appeared unlikely that expressing VgrG-1 from 1587 in a vgrG-1 null mutant of V52 would prohibit Tap-1-mediated secretion of TseL. We, therefore, did not pursue experiment 2.

Instead, we followed the reviewer’s suggestion and carried out experiment 1 in order to differentiate between the effect of a vgrG-1-deletion on Hcp secretion and TseL-mediated killing, as well as mapping VgrG-1 to identify regions required for TseL secretion. In our experiments, full-length VgrG-1 (1163 amino acids in length) and a truncated version missing the C-terminal actin-crosslinking domain (ACD) (1-679) restored TseL-mediated killing of a tsIV1-deletion mutant prey (Figure EV3). An ACD-mutant of VgrG-1 lacking additionally amino acids 642 to 679 abolished TseL-mediated killing. Western-blot analysis of Hcp secretion of the same strains showed that the lack of vgrG-1 resulted in reduced amounts of Hcp secretion compared to wild-type V52. Complementation of the vgrG-1 deletion mutant with any of the three constructs markedly increased Hcp secretion. Combining the two observations, a VgrG-1 construct of amino acids 1-642 restores Hcp secretion but not TseL-mediated killing of a vgrG-1 deletion mutant. Amino acids 642 to 679 connect the gp5-like domain with the actin-crosslinking domain. This 37 amino acid-long sequence is unique to VgrG-1 and not found in VgrG-2 or VgrG-3. We, therefore, conclude that the amino acids 642 to 679 are necessary for VgrG-1 to mediated killing by TseL. We are in the process of testing whether the ability of a truncated VgrG-1 to secrete TseL correlates with its ability to interact with Tap-1.

Based on these results, we added Figure EV3 to the manuscript and modified the text accordingly (l.214ff):

“To identify the domain of VgrG-1 required for TseL-mediated killing, we created truncation mutants and tested the ability of these truncation mutants for TseL-mediated killing and Hcp secretion (Figure EV3A). A truncated version of VgrG-1 missing the C-terminal actin-crosslinking domain (ACD) (1-679) restored TseL-mediated killing and Hcp secretion similar to full-length VgrG-1 (Figure EV3B, C). An ACD-mutant of VgrG-1 lacking additionally amino acids 642 to 679 abolished TseL-mediated killing but maintained Hcp secretion. This 37-amino acid long sequence between position 642 to 679 connects the gp5-like domain with the actin-crosslinking domain of VgrG-1, and is absent from VgrG-2 and VgrG-3 (Figure EV3D). We conclude that amino acids 642 to 679 are necessary for VgrG-1 to mediate killing by TseL.”

2) Experiments showing interactions between Tap-1, VgrG-1 and TseL are well performed and convincing, however, it is not clear if the identified interactions are direct or if additional proteins are involved, such as VgrG-3 (as shown by Dong et al. PNAS 2013). This part of the study would be much stronger if the authors tested the interaction experiments in E. coli. The previous observations made by Dong et al. PNAS 2013 should be discussed in the paper.

We started to further analyze the interactions between Tap-1, VgrG-1 and TseL. Although we have been able to confirm that Tap-1 is required for binding of TseL to VgrG-1 in Vibrio cholerae lysates in additional immunoprecipitation (IP) experiments, our IP studies with recombinant proteins in E. coli did not provide a clear answer as to whether these interactions are direct, or whether they depend on additional proteins. As our efforts to address this question experimentally are ongoing, we added an additional sentence to the discussion to acknowledge that additional proteins might be involved in these interactions.

Discussion (l. 404f):
“To which extend the herein described interactions are direct or depend on additional proteins needs to be further elucidated.”

Regarding the VgrG-3-related claims by Dong et al., we investigated the role of VgrG-3 on secretion of and killing by TseL. We were able to detect TseL secretion and TseL-mediated killing in the absence of VgrG-3. This lead us conclude that VgrG-3, unlike VgrG-1, is not necessary for TseL function. These findings are shown in Figure EV2 and a discussion of the results from Dong et al., PNAS 2013 has been added to the manuscript:

Main text (l. 206ff)

“While VgrG-1 and VgrG-3 differ in their C-termini, they share common core domains that could attract cargo effectors. To test whether VgrG-3 is necessary for TseL function, similar to VgrG-1, we determined the requirement of VgrG-3 for TseL secretion and TseL-mediated killing (Figure EV2). A vgrG-1-deficient mutant was unable to secrete TseL, while secretion of TseL was still observed in a mutant lacking vgrG-3 (Figure EV2A). In the absence of vgrG-3, VgrG-3-mediated killing was abolished, but TseL-mediated killing was still observed (Figure EV2B). Even though VgrG-3 might be involved in the secretion of and killing by TseL, our results indicate that VgrG-3, unlike VgrG-1, is dispensable for TseL function.”

Discussion (l. 405ff):

“A direct interaction between VgrG-3 and TseL has previously been proposed based on the detection of TseL in immunoprecipitates of VgrG-3 (Dong et al, 2013). Our results do not exclude such an interaction, but rather suggest that VgrG-1 is indispensable for TseL whereas VgrG-3 is dispensable.”

3) An experiment that tests for presence of Tap-1 in the supernatant should be part of the main text and at least a supplementary figure should be provided to show the results.

The results of an experiment showing that Tap-1 is retained in the bacterial cell are shown in a new figure, Appendix Figure S1, and discussed in the main text. Additionally, we inserted a reference to the recent paper by Altindis et al., MBio 2015 in which a mass-spec analysis of culture Vibrio cholerae supernatants identified TseL but not Tap-1.

(l. 157 ff)

“Tap-1 was not detected in culture supernatants by us and others, suggesting that Tap-1 is retained in TseL-secreting cells (Appendix Figure S1 and Altindis et al, 2015).”

4) Even though the possible recombination in tap-1 gene is intriguing, the experimental evidence for this is the weakest part of the study and should be either removed or substantially improved. The recombination experiments are poorly described and lack appropriate controls. The paper is interesting and novel enough even without the recombination experiment. If however the authors want to keep this part of the study, more experiments elucidating the mechanism of recombination should be added. Some experiments to consider are for example:

- measurement of recombination efficiency
- dependency on the highly conserved and homologous region of tap-1 as well as other parts of tap-1 and cognate effector genes
- dependency on recA
- test if this is universal for any DNA sequences around the conserved site in tap-1 gene
- Does it depend on other Vibrio genes, or would this happen in E. coli as well?

We thank the reviewer for his/her suggestion to either improve the recombination experiment, or alternatively, remove the experiment from this manuscript. We cloned tap-1 (and a version lacking the core) from strain 1587 into the suicide plasmid pWM91 and conjugated these constructs into Vibrio cholerae C6706 and an isogenic mutant with a transposon insertion in recB. To ensure that we are
looking at differences in recombination frequencies in wild type and the recB mutant, we conjugated the mobilizable plasmid pRP4 as a control (there was no difference: 1/10 wild-type and recB recipients received the plasmid). When we used the recB mutant as the recipient, we detected a ~10-fold drop in conjugation frequencies using lacZ or full-length tap-1 sequences in pWM91. Removing the core region from tap-1 generated numbers close to using pWM91 alone. These results suggest that the core region of tap-1 is recognized as a recombination site, and integration requires the recBCD pathway. However, when we sequenced the tap-1 locus in these conjugants, we found that chromosomal tap-1 was unaffected by the recombination events, suggesting integration of the suicide plasmid into the chromosome by non-homologous recombination. We would like to follow the reviewer’s advice and remove the recombination experiment from the manuscript, so that we can perform additional experiments.

Changes were made to the abstract and the discussion to accommodate for the removal of the recombination experiment:

L. 35 of the abstract now reads:
“Tap-1 contains a conserved core region which if recognized as a recombination site may provide the molecular basis for the exchange of modules.”
(Original sentence l. 34: “We observed a conserved recombination site within tap-1, which may provide the molecular basis for the exchange of modules […]”)

L. 375 of the results now reads:
“Recombination within tap-1 would maintain the open-reading frame…”
(Original sentence l. 349: “Recombination within tap-1 maintained the openreading frame…”)

L. 446 of the discussion now reads:
“Key to the chimeric structure of Tap-1 is the putative recombination site in the middle of tap-1.”
(Original sentence l. 408: “Key to the chimeric structure of Tap-1 is the recombination site in the middle of tap-1.”)

The potential role of the conserved middle region of tap-1 as putative recombination site is now described in the discussion (l. 465ff):
“Any double crossovers that include a complete T6SS cluster like the small auxiliary cluster containing TseL will replace the recipient host cluster with the donor prey cluster. This prey cluster should be functional in the host cell, because the prey cluster provides the proper adaptor gene that operate with the new effector. However, if the recipient carries a VgrG-1 with an actin-crosslinking domain and the prey does not, then the recipient gains a new activity, but loses the ability to crosslink actin. We hypothesize that recombination in the tap-1 core splices a new 3’ end to the existing 5’ end of tap-1 on the chromosome, allowing the cell to use its resident T6SS to secrete newly acquired effectors, while retaining the ACD on VgrG-1.”

Minor concerns:
1) Lines 294-298: It is not completely clear which Tap-1 sequences were analyzed. Figures 5A, C show that class 1 and 2 Tap-1 proteins have very similar N-termini, but class 3 is different (shorter). Were the class 3 Tap-1 proteins part of the alignment in Figure E6 or not? If yes, then how did you deal with the difference in length of class 3 Tap-1 proteins? Please clarify this part of the text and also Figure legends.

The 36 amino acid sequences used for the alignment in Figure 6 (original Figure EV6) contain sequences of Tap-1 from class 1, 2 and 3. For an accurate comparison between the sequences, only the regions present in all three classes of Tap-1 were considered and the first 14 amino acids unique to Tap-1 from class 1 and 2 were excluded.

We are providing a figure in the extended data section (Appendix Figure S5), which shows that the 14 terminal amino acids excluded from the analysis are not required for a functional Tap-1. To clarify which sequences were used in this analysis, we modified the text and figure legends accordingly:
Results:
(L. 333ff)
“Therefore, we analyzed retention of amino acid sequence versus nucleotide sequence by studying the effects of single nucleotide polymorphisms on the amino acid sequences of Tap-1 from all 36 analyzed strains (Figure 5).”
(L. 339ff)
“The short, terminal 14-residue long region of the N-terminal segment unique to class 1 and 2 Tap-1 proteins (Figure 5) was excluded from this analysis, because they would bias the analysis when comparing sequences of class 1 and 2 to class 3 Tap-1 proteins lacking this region. Functional analysis demonstrated that this short region is not required for a functional Tap-1 (Appendix Figure S5), thus supporting our decision to exclude this fragment from our analysis.”

Figure legend of Figure 6 (l. 816f):
“The first 14 amino acids of class 1 and 2 Tap-1 proteins were excluded from this analysis.”
2) Line 297: 61% identity, figure E6 shows 64%
We corrected the sentence accordingly so that it now reads (l. 328f):
“Despite the polymorphic nature of the N-terminal segment of Tap-1, the N-terminal segments of the 36 analyzed sequences still share an overall amino acid sequence identity of 64% (Figure 6A), indicating that they are homologs (Pearson, 2013).”

Referee #2:
The study by Unterweger et al. identified a new adaptor protein, Tap-1, which is required for proper loading of type VI secretion system (T6SS) effector proteins onto the ”weapon tip” of the T6SS tube (e.g., VgrG-associated). The authors used a bioinformatics approach to elucidate the diversity of this protein with respect to it's N-terminal and C-terminal domain and how the diversity mimics the diversity of the up-and down-stream encoded VgrGs and effector proteins, respectively. Indeed, the manuscript convincingly shows that the Tap-1 protein is a chimeric protein. Next, the authors used genetic and biochemical approaches to show that Tap-1 interacts with VgrG-1 and also the effector protein TseL.
Moreover, using immunoprecipitation the authors were able to show that Tap-1 links the VgrG-1 protein and the effector protein TseL and that Tap-1 is specific to VgrG-1 (and not universal for other VgrG proteins that are present in V. cholerae).
In summary, this study is of great importance to better understand the loading of the T6SS system but also to better understand the evolution of V. cholerae strains and how genes encoding tap-1, effector and immunity proteins might be co-transferred between strains to change the predator’s killing / competition potential.
The manuscript is extremely well written and very sound (in fact, I very much enjoyed studying the details of the manuscript). The experiments are well performed and contain all required controls. Altogether this publication will be a great asset to the field and beyond.
There are no major points of critique. Thus, we solely provide minor comments below, which aim at further improving the quality and clarity of the manuscript.
Minor concerns that should be addressed
• Fig. 1: as the authors refer in the text and experiment later on to vasW and vasX it would be good to indicate these genes in Fig. 1C (in addition to the VCA0019 label).

We added the labeling of vasW and vasX to Figure 1C.

• Fig. 3: the authors mention in the text that vgrG-1 deletion leads to less Hcp secretion (as can be seen in the figure). How come this phenotype cannot be complemented with the vgrG-1-carrying plasmid in trans? Does the mutant have a polar effect (e.g., is transcription of the downstream genes affected)? Doesn't seem to be the case though on the TseL levels in the cell pellet)?
We thank the reviewer for bringing this to our attention. In our experiments, we have noticed variations in the levels of Hcp secretion in vgrG-1 deletion mutants complemented with episomal vgrG-1. Any variability in episomal vgrG-1 expression levels (due to differences in arabinose-induced induction) might interfere with restoring Hcp secretion to wild-type levels. Importantly, TseL secretion and TseL-mediated virulence are fully restored in a complemented VgrG-1 mutant despite differences in Hcp levels. It appears that Hcp is not the limiting factor for TseL function, probably because TseL (present at low numbers in the bacterial cytosol) encounters sufficient numbers of Hcp-tubes to be loaded onto even when Hcp-levels are not fully restored. In the western blot shown in Figure 3A, it is important to include Hcp secretion as a control that supernatant was loaded when no TseL could be detected in supernatants.

- Legend to Fig. 4C is unclear. It states that the right part is V52delta-tap-1 and that the strains contained plasmids with bgrG-1-His or FLAG. Thus, in these strains there shouldn't be any Tap-1::FLAG signal at all. We ASSUME that the labeling should be VgrG1::FLAG and VgrG-1::HIS? The authors should correct this or clarify.

We thank the reviewer for bringing this incorrect labeling of the figure to our attention. The reviewer is correct: strains V52 and V52Δtap-1 were transformed with pvgrG-1::HIS and pvgrG-1::FLAG. The signals detected with the anti-FLAG and anti-HIS antibody were subsequently labeled VgrG-1::FLAG and VgrG-1::HIS. Labeling has been corrected.

- Fig. 5A: please mention in the legend what the "+" in the vgrG-1 gene stands for.

The following sentence has been added to the figure legend (l. 789f):
“The presence or absence of the ACD encoded in vgrG-1 is indicated with + and -, respectively.”

- Fig. 5D: As the authors compare all three strains and three Tap1s in panels A, B, C it would be nice to also add all three options (= tap-1V52, tap-1AM-19226, tap-11587) in this experiment (especially as the plasmids are available as shown in panel E). Likewise for panel E (even though parts of the proteins are the same but not the complete one) => at second glance it seems as if that is what the authors did in Fig. E5. Thus, this is redundant and the graphs should be added to the main part.

We would prefer to keep the complete graph in Figure EV5, and show a selection of the tested combinations in Figure 5D and 5E. The selected graphs show one example for the C-terminal segment being specific to its cognate effector, and one example for the N-terminal segment being tolerated by VgrG-1 with or without the ACD. We think that showing one example for each scenario increases clarity.

To connect the text and the figure better and make it more obvious to the reader that additional data can be found in the expanded view figure 5, we added the following link to the main text (l. 299ff):
“To test how differences in the N- or C-terminal segments of Tap-1 affect the function of Tap-1, we compared the adaptor proteins in pairwise combinations (see Figure EV5 for extended analysis of Tap-1 alleles in different strain backgrounds).”

- Fig. 5E: the prey lacks 1501-1503 => please specify (it probably lacks the effector, tap1 homolog and immunity protein-encoding genes, right?)

The reviewer is correct. The following sentence has been added to the figure legend (l. 884f):
“1587ΔA55_1501-03 lacks the genes of auxiliary cluster 1, which encode the
adaptor, effector and immunity proteins.”

• Fig. E5B: as the legend states that Tap-1 expression was looked at by WB it is assumed that the tap-encoding plasmids actually encoded tap-His fusion. This should be indicated in the Figure or at least mentioned in the legend.

Like suggested, we specified the figure legend of this figure (now Figure EV5: (l. 876f)
“Killing assay in which the lack of tap-1 in V52 is complemented by empty vector (control) or one of three different C-terminally HIS-tagged alleles of tap-1.”
(l. 881f)
“Analysis of the expression of the HIS-tagged Tap-1 alleles by SDS-PAGE.”

• Page 16, line 341: please specify the 5’ end and 3’ end of which gene (instead of writing “of C6706 or 1587”)

This sentence has been removed along with other descriptions of the recombination experiment (see response to comment 4 of reviewer 1).

• Fig. E6: tis figure doesn’t provide much new information as the scheme is as the main Fig. 6. The arrows and percentages could easily be added to the main Figure.

Like suggested, we integrated (formerly) Figure E6 into Figure 6.

• Discussion page 18, line 387ff: it is unclear how the detection with the luminolbased chemiluminescent should influence the result? If the protein were unstable in the absence of tap-1 it should be degraded before SDS page and western blotting, right? We suggest deleting this comment.

We think that Tap-1 might be required but not necessary for the stability of TseL. In this case, TseL would still be present in the absence of Tap-1 but in slightly reduced quantities. Small differences in Tap-1 levels would not be detected in our western-blotting based on the non-quantitative nature of luminol-based chemiluminescence.

We clarified our sentence accordingly (l. 426ff):
“Tap-1 may also have a stabilizing function for TseL that we may not have detected due to the non-quantitative nature of luminol-based chemiluminescent detection.”

Additional suggestions for improving the study
• It might be good for the non-expert reader to add the information "auxiliary cluster 2" in Fig. 1C (to make it clear why V. cholerae has two of these clusters). The large cluster does not have a homolog?

Like suggested, “Auxiliary cluster 2” has been added to Figure 1C. The reviewer is correct in that the large cluster does not contain a gene, which encodes a protein of the DUF4123 superfamily.

• Fig. 3: the pairwise statistics isn’t entirely clear; the different between DtsiV2 in V52 versus its vgrG-1 mutant is statistically significant or not?

The difference between surviving C6706ΔtsiV2 exposed to V52 or V52ΔvgrG-1 is statistically significant (unpaired two-tailed t-test, P<0.0001).

For clarification, we are now presenting the results of the killing assays uniformly in Figure 2C, Figure 3C, Figure EV2B, and C.

In the statistical analysis, we now focus on the difference between C6706 and a C6706Δimmunity mutant after exposure to V52. This analysis shows if killing by an effector of interest in V52 is abolished or not.
• Fig. 3B: the labeling isn't very useful as all the names are on top of each other; maybe the font size could be decreased so that the strain names can be recognized?

We assume that the reviewer is referring to Figure 5B instead of 3B. We tried to decrease the font size, which still resulted in overlapping strain names, because the distance between strain names decreased proportionally using FigTree software.
To make the individual strain names visible to the reader, we decided to provide the tree in the layout of a rectangular tree, of which every strain name can be clearly read. This data set has been added as source data for Figure 5D.
Because we think that the layout of the radial tree shows the three clades of Tap-1 the best, we wish to leave this tree in the main figure.

• Ma et al reference => Agrobacterium tumefaciens in italics.

We corrected the layout of the reference accordingly so that it now reads (l. 647ff):

Referee #3:
This manuscript by Unterweger et al. describes experiments that show that Tap-1 functions as an adapter protein that allows effector proteins encoded immediately downstream of tap-1 to be translocated by the T6SS machinery in a manner dependent on the VgrG protein encoded immediately upstream of tap-1. The authors show that Tap-1 binds to both VgrG-1 and TseL individually and simultaneously, but that VgrG-1 and TseL do not bind to each other, providing convincing evidence for their model. By comparing the sequences of Tap-1 homologs in a variety of V. cholerae strains, the authors find that Tap-1 proteins appear to be chimeric and the sequences of their C-terminal segments vary with the sequence of the effector proteins encoded downstream of tap-1. The authors then show that, indeed, the C-terminus of Tap-1 is required for translocation of the specific effector encoded downstream of tap-1. Additional sequence comparisons suggest that highly conserved sequences within tap-1 may be involved in recombination and the authors present an experiment to test this hypothesis. According to the manuscript, the authors transformed strain C6706 with a plasmid containing tap-1 and its downstream effector- and immunityencoding genes from strain 1587, passaged the transformant several times, and then performed PCR using a forward primer that anneals to the 5’ end of tap-1 on the chromosome and a reverse primer that is specific for the 3’ end of the tap-1 allele contained on the plasmid. They sequenced the PCR products and found that the 5’ half (up to the 99 bp of identity between the alleles) of the PCR product corresponded to the C6706 tap-1 gene and the 3’ half corresponded to the 1587 tap-1 allele (the allele on the plasmid). The authors would like to conclude that these results indicate that recombination occurred between tap-1 on the chromosome and tap-1 on the plasmid and that it occurred within the 99 bp conserved region. However, the experiment lacks the important control of demonstrating that the result is not simply a PCR artifact (that is, that they would not obtain the same result even if recombination had not occurred). Moreover, details of the experimental design are lacking. For example, what DNA exactly is contained on the plasmid - specifically, are sequences 3’ to the immunity-encoding gene present? If the chimeric PCR product that the authors obtained is not an artifact, did it result from a single recombination event that occurred at the 99 bp region? Or did a double recombination event occur such that tap-1 plus the effector- and immunity-encoding genes were transferred to the chromosome? It seems that a better experimental design could have been used to determine not only if recombination within the 99 bp can occur, but that it does occur and that the resulting strain with its newly acquired tap-1-effectorimmunity
module has a competitive advantage or an ability to coexist with other strains, as suggested in the abstract.

We thank the reviewer for bringing the potential of PCR artifacts to our attention and for suggesting multiple experiments. We removed the recombination experiment from the current manuscript to test our hypothesis in future experiments (see detailed response to comment 4 of reviewer 1) that we plan to publish in a separate publication.

Minor concerns:
1. Line 29: What do the authors mean by "In our model"?

We modified the indicated sentence (l. 29f):
“Comparison of multiple *V. cholerae* strains indicates that effectors are encoded in modules on mobile genetic elements.”
(The original sentence read:
“In our model, effectors are encoded in modules on mobile genetic elements.”)

2. Many statements throughout the introduction require references. Examples include the sentences ending on lines 46, 51, 52, 57, 74, 86, 88, . . .

As suggested, references have been added to the indicated sentences and now read (references in bold):
Line 47ff:
“One mechanism of competition employed by *V. cholerae* requires direct contact and the type VI secretion system (T6SS) (MacIntyre et al, 2010; Unterweger et al, 2012).”
Line 52ff:
“In the current model for this device, diverse effector proteins are localized to the conserved tip of the inner tube formed of Hcp and are ejected together (Ho et al, 2014; Russell et al, 2014; Zoued et al, 2014).”
Line 54ff:
“Upon delivery of the effector protein into the neighboring cell, the effectors have toxic effects unless inhibited by immunity proteins (Brooks et al, 2013; Dong et al, 2013).”
Line 61ff:
“These effectors are encoded by pandemic *V. cholerae* strains and also by the O37 serogroup strain V52, which has an active T6SS under laboratory conditions (Pukatzki et al, 2006; Unterweger et al, 2014).”
Line 81ff:
“Anti-prokaryotic T6SS effector and immunity proteins are encoded in clusters (Unterweger et al, 2014).”
Line 91ff:
“For example, bacteria of two *V. cholerae* strains with different module sets kill each other in a T6SS-dependent manner (incompatible strains) because they carry different immunity proteins in their effector module sets (Unterweger et al, 2014).”
Line 94ff:
“In contrast, two strains with the same module set do not die when attacking each other, but co-exist (compatible strains) because they carry immunity proteins to the same effectors (Unterweger et al, 2014).”

Additional references have been added to the following sentences:
Line 97ff:
“We have observed that pandemic strains of *V. cholerae* harbour the same T6SS effector module set (Unterweger et al, 2014).”
Line 106ff:
“One class of diverse T6SS effectors are known to contain a conserved PAAR domain for recognition as T6SS substrates (Shneider et al, 2013).”

3. Line 139: "the" should be "they"
The sentence was changed like suggested. The sentence now reads (l. 147ff):
“To determine the secretion requirements for TseL, V52 and an isogenic \textit{tap-1} mutant were maintained in LB broth until they reached the mid-logarithmic phase of growth.”

4. Line 338: "encoding" should be "containing"

This section was modified and does not contain the respective sentence anymore.

5. Lines 359-360: What is the evidence supporting this statement?

We rephrased the sentence so that it now reads (l. 386ff):  
“The proposed mechanism of Tap-1-dependent effector translocation might especially be important for diverse, newly acquired effector proteins, which rely on a conserved T6SS for translocation.”
(Original sentence: "Relying on a conserved T6SS, \textit{V. cholerae} strains utilize Tap-1 to translocate diverse, newly acquired effector proteins.")

6. Lines 505-508: This experiment must be explained in much more detail.

The recombination experiment has been removed from the manuscript (see detailed response to comment 4 of reviewer 1).

2nd Editorial Decision 23 June 2015

Thank you for the submission of your revised manuscript to The EMBO Journal. As you will see below, your article was sent to former referees #1 and #3, who as I mentioned last week, now consider that you have properly dealt with the main concerns originally raised in the review process, and therefore I am writing with an 'accept in principle' decision. This means that I will be happy to formally accept your manuscript for publication once a few more minor issues have been addressed.

As I said, both referees now believe that all major concerns have been addressed and your manuscript is almost ready for publication (see below). Only very minor clarifications raised from referee #3 remain. Browsing through the manuscript myself I have also noticed your very nice statistical descriptions, which I have not been able to find for figure EV3. Could you please add this to the figure legend?

If you have any questions or need any further input, please do not hesitate to contact me.

Thank you very much for your patience. I am looking forward to seeing the final version of your manuscript. Congratulations in advance for a successful publication.

REFEE REPORTS

Referee #1:

All my issues were properly addressed and the study by Unterweger et al. is now suitable for publication.

Referee #3:
The authors have done an excellent job of addressing all of the concerns raised about the initial submission of this manuscript and, in my opinion, the impact of the results is high, even without the recombination experiment that was removed. I have only a few very minor criticisms:

Line 28: Effector translocation is not toxic, the translocated effector in the recipient cell is toxic (unless that recipient cell produces an appropriate immunity protein)

Lines 33-38 - this part of the abstract could be improved for clarity

Lines 56-59: I don't think this is true for all T6S effectors/immunity pairs

Point-by-point response to referees’ comments

Editor

[...] Browsing through the manuscript myself I have also noticed your very nice statistical descriptions, which I have not been able to find for figure EV3. Could you please add this to the figure legend?

Information on the statistical analysis has been added (indicated in bold below). The figure legend now reads (l. 862f):

“(B) Thirty-seven amino acids of VgrG-1 are sufficient to restore TseL-mediated killing. Killing assay in which a tsiV1-deficient mutant of C6706 is exposed to wild-type V52 or V52ΔvgrG-I provided with the indicated constructs of vgrG-I in trans. The arithmetic mean ± SD of log-transformed data of three independent experiments, each performed in duplicate, is shown. P-values of a two-tailed, unpaired t-test are indicated.”

Referee #3:

The authors have done an excellent job of addressing all of the concerns raised about the initial submission of this manuscript and, in my opinion, the impact of the results is high, even without the recombination experiment that was removed. I have only a few very minor criticisms:

Line 28: Effector translocation is not toxic, the translocated effector in the recipient cell is toxic (unless that recipient cell produces an appropriate immunity protein)

As suggested, the sentence was modified and now reads (l. 28f):

“The translocated effectors are toxic unless the targeted cell produces immunity proteins that bind and deactivate incoming effectors.”

(Original sentence: “Effector translocation is toxic unless the targeted cell produces immunity proteins that bind and deactivate incoming effectors.”)

Lines 33-38 - this part of the abstract could be improved for clarity

The indicated sentences were modified and now read (l. 29f):

“Comparison of multiple V. cholerae strains indicates that effectors are encoded in T6SS effector modules on mobile genetic elements. We identified a diverse group of chimeric T6SS adaptor proteins required for the translocation of diverse effectors encoded in modules. An example for a T6SS effector that requires T6SS adaptor protein 1 (Tap-1) is TseL found in pandemic V. cholerae
O1 serogroup strains and other clinical isolates. We propose a model in which Tap-1 is required for loading TseL onto the secretion apparatus. After T6SS-mediated TseL export is completed, Tap-1 is retained in the bacterial cell to load other T6SS machines. “Comparison of multiple V. cholerae strains indicates that effectors are encoded in modules on mobile genetic elements. Effector module sets consist of three modules that differ in their composition among V. cholerae strains. Here, we identified a T6SS adaptor protein, Tap-1, required for translocation of a cognate effector. V. cholerae encodes a diversity of these strain-specific, chimeric Tap-1 adaptor proteins to translocate cognate effectors encoded in module sets. Tap-1 contains a conserved core region which if recognized as a recombination site may provide the molecular basis for the exchange of modules. Thus, the chimeric nature of Tap-1 establishes the molecular basis for the ability of a strain to outcompete or coexist with other strains.”

To increase clarity and to account for the polymorphic nature of the adaptor proteins, the title of the manuscript was also slightly modified and now reads (l. 5):

“Chimeric adaptor proteins translocate diverse type VI secretion system effectors in Vibrio cholerae”

(original title: “Chimeric adaptor protein Tap-1 translocates diverse type VI secretion system effectors in Vibrio cholerae”)

Lines 56- 59: I don’t think this is true for all T6S effectors/immunity pairs

The sentence was modified so that it applies to immunity proteins located in the periplasm and in the cytoplasm (l. 57ff):

“These immunity proteins protect cells from a T6SS-mediated attack by binding and deactivating T6SS effectors (Brooks et al, 2013; Zhang et al, 2014).”

(original sentence: “These immunity proteins are produced in the bacterial cytoplasm and transported across the inner membrane to the periplasm where they protect cells from a T6SS-mediated attack by binding and deactivating the T6SS effectors (Brooks et al, 2013; Zhang et al, 2014).”)

In addition to the changes described above, the following modifications were made to increase clarity:

The following sentence was inserted in l. 49:

Genes of the T6SS are encoded in one large cluster and two auxiliary gene clusters (Pukatzki et al., 2006).

The following sentence replaces two original sentences and now reads (l. 81f):

“Anti-prokaryotic T6SS effector and immunity proteins are encoded in T6SS effector modules that are likely mobile elements exchanged among V. cholerae strains (Unterweger et al, 2014).”

(the original sentences: “Anti-prokaryotic T6SS effector and immunity proteins are encoded in clusters (Unterweger et al, 2014). Differences in GC content and work presented here suggest that these clusters are on mobile genetic elements called T6SS effector modules (Unterweger et al, 2014) that might be exchanged among V. cholerae strains.”)

One sentence was split into two sentences, of which one was moved to the end of the paragraph to increase clarity:

(L. 175f:) “The absence of tap-1 did not abolish killing mediated by VasX or VgrG-3 (Figure 2C).”

(L. 179ff:) “Similarly, a DUF1795 domain in Serratia marcescens also appeared to be specific for a single effector (Diniz & Coulthurst, 2015).”

(original sentence: “The absence of tap-1 did not abolish killing mediated by VasX or VgrG-3 (Figure 2C) similar to what was observed for a T6SS adaptor with a DUF1795 domain in Serratia marcescens (Diniz & Coulthurst, 2015).”)

The following figure legend was modified (insertion in bold) and now reads (l. 821):

“Figure 7. Tap-1 encodes a putative recombination site.”

(original text: Figure 7. “Tap-1 encodes a recombination site.”)