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A binary effector module secreted by a type VI secretion system

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1st Editorial Decision **21st Sep 2021**

Dear Dr. Salomon,

Thank you for transferring your manuscript from Review Commons to EMBO reports. I now went through your manuscript, the referee reports (attached again below), and your revision plan. Both referees acknowledge the potential interest of the findings. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which I see you are willing and able to address during a major revision of the manuscript.

Given the constructive referee comments, we would thus like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response (as suggested in your revision plan). Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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Movies and Datasets should be uploaded separately, using the nomenclature Movie EVx and Dataset EVx. Please provide a legend with title for each movie file as text file and upload it ZIPed together with the movie file. For datasets, please provide a title and legend on the first TAB of the excel file.

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primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates or technical replicates - please clearly indicate this) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This is a clearly-written and presented study describing the function of a new T6SS effector and co-effector. The authors' experimental approaches are careful and thorough as they employ a combination of genetics, genomics, killing assays, protein biochemistry, and microscopy to characterize a binary effector module they initially identified in their model organism (V. para), but is broadly distributed among Vibrionaceae populations that co-occur in the marine environment.

Comments:

1.If a vp1389 deletion results in no detectable expression of the downstream VP1390 effector, this result explains why an immunity mutant is able to survive and is not killed by sibling cell, but that also suggests the interpretation of Fig 2A should be reconsidered, although the conclusions do not change. For example, because the vp1390 deletion does not impact immunity but the vp1389 mutant (which is also functionally a VP1390 null, in theory) is sensitive to killing, one can conclude with confidence that the sensitivity phenotype is due to lack of vp1389. I was initially very confused as to why the vp1389 mutant could survive at all when looking at Fig 2B. The authors could consider moving this information about the polar effect on vp1390 up earlier and incorporating these results into the interpretation of Fig 2A.

2.I was a little confused as to why an in-frame deletion of the internal MIX domain within VP1388 would result in no detectable protein in the western blots. Do the authors think there is some sort of feedback regulation that is dependent on this protein domain or is it a limitation of the approach (ex. antibodies bind to the MIX domain so they are no longer detectable in the VP1388mix- strain). Some sort of speculation might be helpful for the reader here.

3.Lines 85 and 229 - do the authors mean "endogenous" or "in trans" rather than "exogenous" expression/complementation here?

4.Line 210 - typo in Fig 3 legend....should it read "VP1388 binds VP1390" instead of "VP1388 binds VP1388"?

5.The supplemental movies are a really nice addition. However, Movie S2 played fine for me but Movie S1 was unable to play and instead I got a "corrupt file" error.

6.Lines 279-280 - do the authors have some representative still images to show the blebs forming at the septum as described in the results section? I was looking for them in Fig 5 and the supplemental movie and it was hard to see.

7.Lines 311-315 - I appreciate the authors disclosing that they have attempted to characterize the secretion mechanisms but I found the added details about it being "sticky" to be a little confusing and probably not necessary. As a reader, I would be more than satisfied to know attempts were made and so far are inconclusive. The authors could consider trimming this sentence to just say...."Nevertheless, although we have made numerous attempts to decipher the molecular mechanism that is used by VP1388 to enable VP1390 secretion, the results have been inconclusive and prevent us from shedding more light on the mechanism in detail at this stage."

8.Figure 6 - in the model, which is very nicely drawn, it looks like the immunity protein is interacting with the PG. Is this on purpose because the authors predict/know VP1389 interacts with the PG, perhaps because they predict that the VP1390 toxin targets PG, or does the immunity protein just overlap with PG in the drawing due to space limitations in the figure?

9.The authors mention in the introduction and discussion that vibrios are emerging pathogens to humans and animal and reference Horseman et al 2013. The authors could consider expanding on this to include more recent references (for example PMID: 3002421 for human pathogens). Many of the species listed in the supplemental table that have homologs of this newly described binary effector module are in pathogens (or beneficial symbionts) of humans and animals and specifically noting those species they found could further underscore the importance of this new system in competition for colonization sites across diverse hosts.

10.Line 441 - the authors describe how they performed immunoblots to assay protein levels in V. para and describe using phenamil to induce T6SS expression in liquid, but in the main text for this experiment and the description of other similar methods the hns deletion mutant alone was used to induce T6SS expression in liquid. Is it necessary to use phenamil supplementation and hns mutant strains to induce T6SS expression/secretion in liquid or is one approach sufficient?

Significance:

This work expands our general understanding of how a common secretion system translocates effectors. The findings described here are significant because they assign a new function to a broadly-conserved, T6SS-associated protein (the co-effector), and underscore the importance of exploring how T6SS effectors may be exported out of the cell by previously unknown mechanisms. This work will be of interest to researchers working broadly on secretion systems, especially the type VI secretion system (my particular field), as well as research groups studying current and emerging vibrio pathogens.

Referee #2:

The manuscript by Dar et al., describes a novel role for a previously uncharacterized protein in Vibrio parahaemolyticus. The group aimed to decipher the role that a tricistronic operon plays in antibacterial competition of V. parahaemolyticus. The group had previously demonstrated that VP1389 is the immunity protein to an unknown effector protein within the pathogenic T6SS1 locus of V. parahaemolyticus. However, little was known about the function of the other two genes vp1388 and vp1390 within this operon. The group now demonstrates that VP1390 is the toxic effector protein to the immunity protein VP1389 and that VP1388 and VP1390 directly interact with another to get loaded onto the spike protein of the T6SS. The secretion of VP1388, containing the MIX domain for effector secretion, and VP1390 are co-dependent on one another. The authors also provided data on the effect of VP1390 toxin on prey cells.

The manuscript appears technically sound. Experiments were performed with appropriate rigor and, with exceptions noted below, included relevant controls. In addition, the manuscript is well written, has good rationale, follows a clear logic, and features easy to understand and clear graphs. The presented experiments support the authors' hypothesis that VP1390 is the toxic effector protein and requires VP1388 for secretion. One shortcoming of this paper is that the effector has not been shown to work in an actual inter-bacterial competition. Literature holds examples for that overexpression of effectors in target bacteria can produce results that are not a reflection of results achieved by natural effector translocation. However, even without this data the novel effector translocation mechanism is very interesting.

The following control experiments are required to support the authors' claims. Furthermore, some additional experiments would strengthen the data presented in this manuscript.

1.The authors very nicely verified the toxic effects of VP1390 using interbacterial competition assays and exogenous expression in another host and microscopy images including various controls. The same rigor is not applied to microscopy studies in Fig. 5. Here, a control group of deletion mutant in vp1390 is missing. The observed effect could be mediated by other effectors (Figure 2C clearly demonstrates that other than VP1390, there are other effector proteins that kill). Therefore, in addition to a T6SS deficient mutant, authors should use a vp1390 mutant.

2.The authors performed their E. coli assays at 30C. However, the microscopy images for E. coli were taken at 37C. Could the increased temperature influence the function of the toxin? Ideally, the authors would confirm their findings at 30C. Minimally, they should provide rationale for why the experiments were performed at elevated temperatures.

3.The E. coli data seem to confirm that VP1390 is a toxin. However, according to the authors the expression of the immunity protein is also toxic in E. coli, casting some doubt on the strength of the supplied data. If a non-toxic immunity protein kills E. coli, how specific are the effects of the actual toxin that are observed in E. coli? Have the authors tried to reduce expression level of the immunity protein?

4.The authors nicely show in E. coli that VP1390 directed to the periplasm results in death of E. coli cells. However, the control experiments showing that "misguided" VP1390 does not kill are missing. Do E. coli cells expressing VP1390 in the cytoplasm remain viable and is a V. parahaemolyticus strain expressing VP1389 without the periplasm targeting signal sensitive to killing? These experiments are not absolutely required, but authors should soften their wording on that targeting to the periplasm is required in case they will not be performed.

Minor:

1.In Supplementary Video 1, the expression of vp1390 not only results in PI uptake and lysis, but cells are also dividing slower than the control groups prior to PI uptake. This seems to suggest that the toxin is interfering in growth even before cells die. The authors should expand on this observation in their discussion section.

2.Fig. 3 legend should likely read "A) VP 1388 binds to VP1390" or vice versa.

3.Rationale for using hns background strains could be stronger. Killing seems to be as efficient by WT (Fig. 2A) compared to hns strains (Fig. 2B).

Significance:

The most significant aspect of this manuscript is the novelty of the tricistronic operon. The authors present a new concept of how effectors can be loaded onto the T6SS. The findings are most relevant to researchers studying T6SS and to researchers studying related bacterial competition systems. The T6SS research field is constantly discovering new effectors and effector functions and this manuscript adds a novel form of effector delivery. The authors clearly demonstrate a new interbacterial effector plus mediator protein transport system. They provided clear data that show both the effector and its co-effector are loaded together onto the T6SS apparatus. The MIX domain for effector secretion is present in the VP1388 and not in the actual effector VP1390. The group also demonstrated that VP1388 protein is unlike previously known chaperon proteins. Traditionally,

chaperon proteins are not secreted with their cognate effector proteins. However, this manuscript provides the field with new information on how an effector protein requires this new co-effector protein for its secretion and function in the target bacterium.

Expertise keywords: Enterobacteriaceae, interbacterial competition mechanisms. Less familiar with the bioinformatic aspects of the manuscript.

Point-by-point response to reviewers

Referee #1:

This is a clearly-written and presented study describing the function of a new T6SS effector and co-effector. The authors' experimental approaches are careful and thorough as they employ a combination of genetics, genomics, killing assays, protein biochemistry, and microscopy to characterize a binary effector module they initially identified in their model organism (V. para), but is broadly distributed among Vibrionaceae populations that co-occur in the marine environment.

We thank the reviewer for this kind assessment of our work.

Comments:

1.If a vp1389 deletion results in no detectable expression of the downstream VP1390 effector, this result explains why an immunity mutant is able to survive and is not killed by sibling cell, but that also suggests the interpretation of Fig 2A should be reconsidered, although the conclusions do not change. For example, because the vp1390 deletion does not impact immunity but the vp1389 mutant (which is also functionally a VP1390 null, in theory) is sensitive to killing, one can conclude with confidence that the sensitivity phenotype is due to lack of vp1389. I was initially very confused as to why the vp1389 mutant could survive at all when looking at Fig 2B. The authors could consider moving this information about the polar effect on vp1390 up earlier and incorporating these results into the interpretation of Fig 2A.

To clarify the point raised by the reviewer, we addressed the polar mutation found in the vp1389 deletion strain in the appropriate section describing the results in Fig. 2A (lines 140-144): "Surprisingly, VP1390 expression was absent in the Δ*vp1389* mutant (Fig. EV2A). We reasoned that this deletion resulted in a polar effect; if VP1390 plays a role in antibacterial toxicity, as investigated below, then this polar effect could explain how we were able to obtain a mutation in a putative immunity gene without it being killed by its neighboring kin."

2.I was a little confused as to why an in-frame deletion of the internal MIX domain within VP1388 would result in no detectable protein in the western blots. Do the authors think there is some sort of feedback regulation that is dependent on this protein domain or is it a limitation of the approach (ex. antibodies bind to the MIX domain so they are no longer detectable in the VP1388mix- strain). Some sort of speculation might be helpful for the reader here.

Indeed, we were pondering this while analyzing the results. The custom-made antibody's recognition site is not part of the deleted region (see Methods section "Endogenous expression of VP1388 and VP1390 in V. parahaemolyticus"), and therefore it is probably

not a matter of detection. Without further evidence, we speculate that the deletion could affect the stability of either the transcript or the protein. We have added a note addressing this in the text (lines 157-160): “Since the antibody used to detect VP1388 expression recognizes an epitope found outside of the deleted region, we suspect that this deletion affected the stability of the *vp1388* transcript or the protein, thus resulting in lack of detectible protein expression.”

3.Lines 85 and 229 - do the authors mean "endogenous" or "in trans" rather than "exogenous" expression/complementation here?

We thank the reviewer for pointing out this mistake. Indeed, this should be expression “in trans”. This was amended in the revised version.

4.Line 210 - typo in Fig 3 legend....should it read "VP1388 binds VP1390" instead of "VP1388 binds VP1388"?

We thank the reviewer for alerting us to this error. The mistake was corrected.

5.The supplemental movies are a really nice addition. However, Movie S2 played fine for me but Movie S1 was unable to play and instead I got a "corrupt file" error.

We apologize for this inconvenience. The difference is due to the fact that the movies are not the same file type (movie S1 is an MP4 file whereas movie S2 is an AVI file). This may result in some media players playing one file but not the other. We will consult with the production team before publication to decide which format is preferable.

6.Lines 279-280 - do the authors have some representative still images to show the blebs forming at the septum as described in the results section? I was looking for them in Fig 5 and the supplemental movie and it was hard to see.

Representative images are provided in Figure EV5.

7.Lines 311-315 - I appreciate the authors disclosing that they have attempted to characterize the secretion mechanisms but I found the added details about it being "sticky" to be a little confusing and probably not necessary. As a reader, I would be more than satisfied to know attempts were made and so far are inconclusive. The authors could consider trimming this sentence to just say...."Nevertheless, although we have made numerous attempts to decipher the molecular mechanism that is used by VP1388 to enable VP1390 secretion, the results have

been inconclusive and prevent us from shedding more light on the mechanism in detail at this stage."

We thank the reviewer for this comment. The text was revised as suggested by the reviewer (lines 278-281).

8.Figure 6 - in the model, which is very nicely drawn, it looks like the immunity protein is interacting with the PG. Is this on purpose because the authors predict/know VP1389 interacts with the PG, perhaps because they predict that the VP1390 toxin targets PG, or does the immunity protein just overlap with PG in the drawing due to space limitations in the figure?

We do not have information relating to the exact location of VP1389 in the periplasm, beyond the presence of an N-terminal signal peptide. The mentioned overlap with the peptidoglycan in the model is simply due to space constrains.

9.The authors mention in the introduction and discussion that vibrios are emerging pathogens to humans and animal and reference Horseman et al 2013. The authors could consider expanding on this to include more recent references (for example PMID: 3002421 for human pathogens). Many of the species listed in the supplemental table that have homologs of this newly described binary effector module are in pathogens (or beneficial symbionts) of humans and animals and specifically noting those species they found could further underscore the importance of this new system in competition for colonization sites across diverse hosts.

We thank the reviewer for these suggestions. We have added additional citations (lines 63-64) and noted several specific Vibrio strains of interest that carry the new binary effector module in the text (lines 108-112): "Many of these bacteria are known or emerging pathogens of humans and marine animals, such as *V. parahaemolyticus* (Letchumanan et al, 2014), *V. vulnificus* (Baker-Austin & Oliver, 2018), *V. coralliilyticus* (Ben-Haim et al, 2003), *V. harveyi* (Zhang et al, 2020), and *V. crassostreae* (Bruto et al, 2017); some are symbionts of marine animals, such as *Allivibrio fischeri* (Visick et al, 2021)."

10.Line 441 - the authors describe how they performed immunoblots to assay protein levels in V. para and describe using phenamil to induce T6SS expression in liquid, but in the main text for this experiment and the description of other similar methods the hns deletion mutant alone was used to induce T6SS expression in liquid. Is it necessary to use phenamil supplementation and hns mutant strains to induce T6SS expression/secretion in liquid or is one approach sufficient?

Deletion of *hns* in *V. parahaemolyticus* de-represses T6SS1 expression and activity, and therefore it negates the need to induce surface sensing by adding phenamil to liquid media. Indeed, phenamil was not added in all experiments in which an *hns* deletion background was used. However, the Methods section mentioned by the reviewer (Endogenous expression of VP1388 and VP1390 in *V. parahaemolyticus*) describes the

methodology used to perform the experiment shown in Figure EV2A. In this experiment, the strains did not contain an *hns* deletion, and therefore phenamil was used to induce T6SS1 in liquid.

Significance:

This work expands our general understanding of how a common secretion system translocates effectors. The findings described here are significant because they assign a new function to a broadly-conserved, T6SS-associated protein (the co-effector), and underscore the importance of exploring how T6SS effectors may be exported out of the cell by previously unknown mechanisms. This work will be of interest to researchers working broadly on secretion systems, especially the type VI secretion system (my particular field), as well as research groups studying current and emerging vibrio pathogens.

We thank the reviewer for kindly summarizing the significant of this work.

Referee #2:

The manuscript by Dar et al., describes a novel role for a previously uncharacterized protein in Vibrio parahaemolyticus. The group aimed to decipher the role that a tricistronic operon plays in antibacterial competition of V. parahaemolyticus. The group had previously demonstrated that VP1389 is the immunity protein to an unknown effector protein within the pathogenic T6SS1 locus of V. parahaemolyticus. However, little was known about the function of the other two genes vp1388 and vp1390 within this operon. The group now demonstrates that VP1390 is the toxic effector protein to the immunity protein VP1389 and that VP1388 and VP1390 directly interact with another to get loaded onto the spike protein of the T6SS. The secretion of VP1388, containing the MIX domain for effector secretion, and VP1390 are co-dependent on one another. The authors also provided data on the effect of VP1390 toxin on prey cells.

The manuscript appears technically sound. Experiments were performed with appropriate rigor and, with exceptions noted below, included relevant controls. In addition, the manuscript is well written, has good rationale, follows a clear logic, and features easy to understand and clear graphs. The presented experiments support the authors' hypothesis that VP1390 is the toxic effector protein and requires VP1388 for secretion. One shortcoming of this paper is that the effector has not been shown to work in an actual inter-bacterial competition. Literature holds examples for that overexpression of effectors in target bacteria can produce results that are not a reflection of results achieved by natural effector translocation. However, even without this data the novel effector translocation mechanism is very interesting.

We thank the reviewer for this kind assessment of our work. We agree with the reviewer's note that a direct role of VP1390 alone in bacterial competition would have further strengthen the claims of VP1390 being the toxin. Nevertheless, the nature of secretion

via this binary effector module mechanism prohibited us from performing such a direct assay, since VP1390 delivery is strictly dependent on VP1388, which is also secreted.

The following control experiments are required to support the authors' claims. Furthermore, some additional experiments would strengthen the data presented in this manuscript.

1.The authors very nicely verified the toxic effects of VP1390 using interbacterial competition assays and exogenous expression in another host and microscopy images including various controls. The same rigor is not applied to microscopy studies in Fig. 5. Here, a control group of deletion mutant in vp1390 is missing. The observed effect could be mediated by other effectors (Figure 2C clearly demonstrates that other than VP1390, there are other effector proteins that kill). Therefore, in addition to a T6SS deficient mutant, authors should use a vp1390 mutant.

As the reviewer suggested, we added a Δ*vp1390* strain as an attacker to the microscopy experiment shown in Fig. 5. As expected, the Δ*vp1390* attacker was unable to intoxicate Δ*vp1389* prey, similar to the T6SS1$^-$ Δ*hcp1* attacker. See revised Results section (lines 246-258) and revised Fig. 5.

2.The authors performed their E. coli assays at 30C. However, the microscopy images for E. coli were taken at 37C. Could the increased temperature influence the function of the toxin? Ideally, the authors would confirm their findings at 30C. Minimally, they should provide rationale for why the experiments were performed at elevated temperatures.

The spotting assay shown in Fig. 4A was performed at 30°C because of a technical preference; the slower growth at the lower temperature allowed us to monitor their overnight growth better. We have now replaced this experiment with one performed at both 30°C and 37°C in parallel. As shown in the new panel (Fig. 4A), the same results are obtained when cells are incubated at either temperature, i.e., periplasmic VP1390, but not VP1388, is toxic upon expression in *E. coli*. The *E. coli* microscopy was performed at 37°C since this is the optimal temperature for *E. coli* growth over a short time frame.

3.The E. coli data seem to confirm that VP1390 is a toxin. However, according to the authors the expression of the immunity protein is also toxic in E. coli, casting some doubt on the strength of the supplied data. If a non-toxic immunity protein kills E. coli, how specific are the effects of the actual toxin that are observed in E. coli? Have the authors tried to reduce expression level of the immunity protein?

The competition assays in *Vibrio* clearly demonstrate that either VP1388 or VP1390 is the toxin. Based on this premise, the interpretation of the *E. coli* toxicity results was made. Nevertheless, we agree with the reviewer that over-expression in *E. coli* as sole evidence is insufficient to ascribe the role of toxin to a protein. For this reason, we include as supporting evidence the direct interaction of VP1390 with the immunity protein, VP1389 (Fig. 4B). This link between the two proteins is further strengthened by the presence of VP1389-VP1390 "dyads" in many bacteria lacking a VP1388 homolog (Fig. 1B). Another

supporting result is the lysis phenotype that is observed in both sensitive vibrios during competition (Fig. 5) and in *E. coli* (Fig. 4C) upon expression of periplasmic VP1390, but not upon expression of periplasmic VP1388. We tried to reduce the expression of VP1389, but some toxicity remained when expressed in *E. coli* even at low arabinose concentrations. At very low concentration, VP1389 is no longer toxic, but we cannot confirm its expression.

4.The authors nicely show in E. coli that VP1390 directed to the periplasm results in death of E. coli cells. However, the control experiments showing that "misguided" VP1390 does not kill are missing. Do E. coli cells expressing VP1390 in the cytoplasm remain viable…

To address the reviewer's question, we added Fig. EV4D,E showing that a cytoplasmic version of VP1390 is dramatically less toxic than a periplasmic version of VP1390. See lines 227-229: "Notably, Expression of VP1390 in the *E. coli* cytoplasm (without a PelB signal peptide) proved dramatically less toxic compared to the detrimental effect caused by expression of periplasm-destined VP1390 (Fig. EV4D,E)."

…and is a V. parahaemolyticus strain expressing VP1389 without the periplasm targeting signal sensitive to killing? These experiments are not absolutely required, but authors should soften their wording on that targeting to the periplasm is required in case they will not be performed.

To address the reviewer's question, we added Fig. EV4A,B showing that a VP1389 mutant lacking the N-terminal signal peptide is unable to provide protection against T6SS1-mediated attacks, compared to wild-type VP1389. This result supports the notion that the toxic activity mediated by the binary toxin module takes place in the periplasm. See lines 217-223: "To support this premise, we tested whether VP1389 lacking its predicted signal peptide (VP1389$^{\Delta SS}$, amino acids 18-375; predicted by SignalP-5.0 (Almagro Armenteros *et al*, 2019)) can protect a Δ*vp1389* prey against a T6SS1-mediated attack. As shown in Fig. EV4A, removal of the signal peptide abrogated VP1389's ability to antagonize a T6SS1-mediated attack during bacterial competition, even though both the wild-type VP1389 and VP1389$^{\Delta SS}$ appear to express at a comparable level (Fig. EV4B). This result suggests that the toxic activity mediated by either VP1388 or VP1390 occurs in the prey periplasm".

Minor:

1.In Supplementary Video 1, the expression of vp1390 not only results in PI uptake and lysis, but cells are also dividing slower than the control groups prior to PI uptake. This seems to suggest that the toxin is interfering in growth even before cells die. The authors should expand on this observation in their discussion section.

As suggested by the reviewer, we now describe this observation in the Results section (lines 241-242): "We also observed that cells expressing the periplasmic VP1390 divide

slower than cells expressing VP1388 or sfGFP; this was apparent even prior to PI entry (Movie. EV1).", and in the Discussion section (lines 298-300): "Notably, *E. coli* cells expressing periplasmic VP1390 divided slower than their control counterparts, suggesting that VP1390 affects the cell even before PI uptake and lysis occur."

2.Fig. 3 legend should likely read "A) VP 1388 binds to VP1390" or vice versa.

We thank the reviewer for alerting us to this error. The mistake was corrected.

3.Rationale for using hns background strains could be stronger. Killing seems to be as efficient by WT (Fig. 2A) compared to hns strains (Fig. 2B).

We wanted to use the *hns* background because it provides a more 'stable' phenotype for subsequent secretion assays than using externally added phenamil, which may have some fluctuations in the amplitude of T6SS induction between experiments. We now clarify the rationale in the text (lines 170-174): "Notably, the attacker strains that were used for these assays were generated in a background in which *hns*, encoding a negative regulator of T6SS1 (Salomon *et al*, 2014b; Fridman *et al*, 2020), was deleted (Δ*hns*) to ensure maximal activation of T6SS1 and to eliminate possible fluctuations between experiments in the amplitude of T6SS1 activation by external conditions."

Significance:

The most significant aspect of this manuscript is the novelty of the tricistronic operon. The authors present a new concept of how effectors can be loaded onto the T6SS. The findings are most relevant to researchers studying T6SS and to researchers studying related bacterial competition systems. The T6SS research field is constantly discovering new effectors and effector functions and this manuscript adds a novel form of effector delivery. The authors clearly demonstrate a new interbacterial effector plus mediator protein transport system. They provided clear data that show both the effector and its co-effector are loaded together onto the T6SS apparatus. The MIX domain for effector secretion is present in the VP1388 and not in the actual effector VP1390. The group also demonstrated that VP1388 protein is unlike previously known chaperon proteins. Traditionally, chaperon proteins are not secreted with their cognate effector proteins. However, this manuscript provides the field with new information on how an effector protein requires this new co-effector protein for its secretion and function in the target bacterium.

We thank the reviewer for kindly summarizing the significant of this work.

Dear Dr. Salomon,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of the manuscript. Referee has one further suggestion you might want to address in the final revised version.

Moreover, I have these few editorial requests:

- Please provide the abstract written in present tense throughout.

- Please check that Biorender.com is properly cited (depending on your subscription and templates used). See: https://help.biorender.com/en/articles/3619405-how-do-i-cite-biorender

- Please remove the movie legends from the main manuscript text. Please provide these as text files, ZIPed together and uploaded with the movie file.

- Please also remove the legends for the EV datasets. Please put these on the first TAB of the dataset excel files.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with very few changes and queries we ask you to include in your final manuscript text.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have carefully revised their manuscript based on our comments during the review process in review commons. They addressed all our concerns by adding relevant control experiments (for Fig. 4 and 5) and by performing additional experiments to support their conclusions (Fig. EV2 and EV4). The expression of VP1390 in the cytoplasm very convincingly supports their hypothesis that it is active in the periplasm of target cells. This is also supported by their data showing that a VP1389 mutant lacking the N-terminal signal peptide is unable to provide protection.
The reply to our query of why the immunity protein expressed in E. coli is toxic also seems sufficient. The authors might want to consider adding that they attempted to reduce toxicity of the immunity protein to their manuscript, but this would not be absolutely required.

Referee #2:

The authors have provided adequate responses to the reviewer critiques and the revised manuscript is a strong and important contribution to the field. This reviewer does not have any further comments for the authors to consider.

Dear Dr. Breiling,

We thank you for inviting us to submit a final revised version of our manuscript EMBOR-2021-53981V2 entitled “A binary effector module secreted by a type VI secretion system”. We have addressed the requested revisions and we believe that the manuscript is now suitable for publication in *EMBO reports*.

Changes included in the final revised version:

- Abstract is now written in present tense.
- Biorender is now appropriately cited in the Acknowledgements section (purple text).
- Movie legends were removed from the main text and are provided as text files ZIPed together with the movie files.
- Legends for the EV databases were removed from the main text and are found in the first tab of the excel files.
- Publisher changes in the main text file were addressed (purple text).
- As for the optional request by reviewer #1 to add the attempts to reduce the toxicity of the immunity protein in *E. coli* – we prefer not to include this information in the text. We believe that since this is a subjective statement that will not be backed by actual data or results, it will not add to the readers’ understanding of the manuscript. Moreover, our reply to the reviewer’s original query will be included in the summary of the editorial decision process that will be available to readers. If the editor disagrees with us, then we will of course reconsider.

Dr. Dor Salomon
Tel Aviv University
Clinical Microbiology and Immunology
Ramat Aviv
Sackler Faculty of Medicine
Tel Aviv 6997801
Israel

Dear Dr. Salomon,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Corresponding Author Name: Dor Salomon
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2021-53981V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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Is the variance similar between the groups that are being statistically compared?	NA

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).	1) Anti-Myc antibody: Santa Cruz Biotechnology, 9E10, mouse mAb, sc-40; 2) Anti-FLAG antibody: D6W5B rabbit mAb #14793; Cell Signaling Technology; 3) Anti-VP1388 antibody: Rabbit poly-clonal antibody raised against peptide CLAEDLQPVDKETQM by Genscript. Specificity shown in Figure 3B and Figure EV2A ; 4) Anti-VP1390 antibody: Rabbit poly-clonal antibody raised against peptide EDENNDKTYPSWHSC by Genscript. Specificity shown in Figure 3B and Figure EV2A ; 5) Anti-VgrG1 antibody: Li, P. et al. Acute aepatopancreatic necrosis disease-causing Vibrio parahaemolyticus strains maintain an antibacterial Type VI secretion system with versatile effector repertoires. Appl. Environ. Microbiol. 83, e00737-17 (2017).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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E- Human Subjects

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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The authors declare that all data supporting the findings reported here are available within the manuscript and its associated files. No primary datasets were generated or deposited during this study.
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