EMBO reports

Engineering a customizable antibacterial T6SS-based platform in Vibrio natriegens

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1st Editorial Decision 23rd Jul 2021

Dear Dr. Salomon,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript and the referee reports from The EMBO Journal (attached again below). Both referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, we feel all need to be addressed during a major revision.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. 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.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

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. 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.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) 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:
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9) Please provide the abstract written in present tense add up to 5 key words to the title page.

10) Please note our new reference format:
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11) Please add a paragraph detailing the author contributions to the manuscript. Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - DAS - Acknowledgements - Author contributions - Conflict of interest - References - Figure legends - Expanded View Figure legends.

12) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

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.

Kind regards,

Achim

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this manuscript, the authors describe their success in moving T6SS gene cluster from Vibrio parahaemolyticus to Vibrio natriegens. They show that the T6SS is fully functional in V. natriegens and that the transformed bacteria gained an ability to kill bacterial prey. In addition, the authors show that this system has a potential to be used with various antibacterial toxins. Authors also rendered T6SS expression and activity inducible by putting its regulator under an arabinose inducible promoter. The manuscript is well written, figures properly show results of all experiments and the right controls were performed throughout.
While the manuscript is solid, I fail to see its impact beyond showing a potential for engineering of strains that could be used under some specific conditions to eliminate some bacteria. It is an interesting application of what we know about T6SS but everything presented here is somewhat expected and brings no new insights into how the system works or what is its role during bacterial interactions. I think that the potential for using the developed strain to learn more about uncharacterized T6SS effectors is not clearly shown by the authors. Therefore, I would expect to see this type of manuscript in a specialized biotechnological journal. I don't mean to be negative about the work itself, I just don't see it as a good fit for the EMBO Journal.

In addition, I would say that the Figure 1 clearly shows that the T6SS in V. natriegens is expressed less and is less active than in Vibrio parahaemolyticus. This means that there is a room for major improvement of the system by more engineering, tweaking the expression level of the system. Unfortunately Figure 4 also highlights how difficult it will be to use this approach to eliminate certain bacteria from a complex mixture. The attackers had to be present in a ten-fold excess to achieve detectable decrease in abundance of certain species. This is of course also dependent on the sensitivity of the target cells and thus could be potentially improved by a careful selection of more potent toxins. Nevertheless, this experiment shows that the major limitation of using T6SS to kill bacteria is that the attacker and the prey cells have to be in a close contact and thus the attacker has be present in a significant amount to increase the chance that the attacker meets and kills its prey.

Referee #2:

The Type 6 Secretion System (T6SS) is a syringe-like apparatus, used by some bacteria to inject toxins into other cells. T6SS-wielding bacteria could be used to attack pathogens, offering an alternative to conventional antibacterials. To this end, the authors engineer a T6SS+ attacker strain that can deliver customisable toxin payloads to various target bacteria. Their approach demonstrates several significant advances:

1) The attacker strain can be a non-pathogen. In nature, T6SSs are often found in pathogenic bacteria. Instead of using such a pathogen, the authors transplant a T6SS operon (via plasmid) into non-pathogenic V. natriegens, reducing the risk of harm to host organisms during therapy.

2) T6SS activity is controlled externally. By identifying a T6SS regulator protein (VP1407) and linking its expression to an external inducer (arabinose), the authors demonstrate that T6SS expression and killing can be controlled by tuning environmental conditions.

3) T6SS effector payload can be freely altered, to deliver single or multiple toxins of a user's choosing. This allows the toxin arsenal to be tailored to target different bacteria.

The end result is a flexible platform for delivering configurable T6SS effectors into diverse target bacteria, under various temperature conditions, all under the control of an exogenous inducer. The modular design (effector repertoire and regulation system configurable via plasmid choice) make this platform easily customisable, while preventing unintentional proliferation of T6SS weapon genes via horizontal gene transfer.

Assessment:

The paper demonstrates multiple significant advances towards the goal of harnessing T6SS-armed bacteria as biocontrol agents or biotherapeutics. The V. natriegens platform also offers immediate applications as a system for profiling T6SS effector potency / synergy, as well as for studying prey susceptibility factors and resistance. The paper is technically sound and thorough throughout, with appropriate controls and statistical analyses included at every stage. The paper is also excellently structured: it sets out clear, well-motivated goals, and addresses them systematically. The writing and figures are easy to follow.

Given the high quality of this work and its importance for the field, I strongly recommend that this paper be accepted for publication. I have a few suggestions for improving readability, but these are non-essential.

General:

1)To measure T6SS-mediated killing, the authors conduct competition assays using an excess of attackers (4:1 attacker:prey, or 10:1 vs prey cultures). This is common practice and fine in itself - I understand the need to create strong killing effects for detection by the (low precision) CFU counting method. However, I feel that the authors should acknowledge that the competition conditions are somewhat idealised in favour of the attacker, and may not be representative of competition conditions in nature.

2) T6SS effectiveness is famously poor in liquid culture, as cell-cell contact is transient. How will this impact the effectiveness of the V. natriegens platform as a biocontrol agent in aquaculture systems? I am not sure to what extent competition will be surficial in this context; could the authors clarify? It might also be worth pointing out that the nanobody adhesion system of Ting et al. makes T6SS killing effective in liquid culture, so - in combination with this work - this could be a good way of extending the conditions in which V. natriegens is an effective killer (as well as improving specificity, as noted on L323).

Specific:

L56: I think 'lucrative' is misleading here since (notwithstanding the authors' patent application) this system has yet to be commercialised. I suggest 'promising' as an alternative.

L144 and Fig 2B: I do not understand why you label the vp1407 knockout pT6SS1Ind. To me, 'Ind' suggests induction, but surely the point here is that there's no induction because a key positive regulator has been removed. Maybe pT6SS1Δvp1407 would be more intuitive?

Fig 2C: I got confused comparing this to Fig 1B. In 1B, you use asterisks to point out additional non-specific bands that appear in V. natriegens samples, which don't correspond to VgrG1. Are these bands present in Fig. 2C (and if not, why not)? I initially interpreted 2C as indicating no T6 secretion

occurred under any conditions, but in fact you're showing that firing only occurs if i) there's a functional T6SS present, and ii) the arabinose inducer is provided. Is that correct? Overall, I think you just need to clarify why the extra, non-VgrG1 bands present in Fig. 1B disappear in Fig. 2C.

Fig 5C: The text and cell colours are very faint; making both larger and using bolder colours would improve readability here.

L 328: What is the rationale for having the exogenous effector/immunity genes controlled by the same regulator as the T6SS core? Is this a cost-reduction measure?

L349. The ability to study single effectors in isolation makes sense as an application of your system. However, comparing this with exogenous toxin expression in prey cells seems like a bit of a straw man - does anyone actually use this to assess potency?

L349. Relating to the above: surely the key advantage of your platform (over, say, just knocking out all but one effector in a T6SS+ bacterium) is that you can compare effectors from lots of different species in a single platform, where T6SS and effector expression are tightly (and externally) controlled? This seems like a great way of removing confounding factors (e.g. species A making more effector / firing faster than species B) from effector comparisons. I think it would be worth pointing this advantage out.

I would like to close by thanking the authors for their work - it makes for a very exciting read. I wish them the best of luck with their forthcoming work.

Point-By-Point response to reviewer comments

Referee #1:

- The manuscript is well written, figures properly show results of all experiments and the right controls were performed throughout.

  We thank the reviewer for acknowledging this.

- I fail to see its impact beyond showing a potential for engineering of strains that could be used under some specific conditions to eliminate some bacteria

  We strongly believe that beyond what the reviewer stated, this work has additional impact. We are convinced that this will be of interest to a large community outside the T6SS field.
  1) **It provides a methodology on how to study T6SSs and single/multiple effectors from bacteria that may be unavailable/unculturable or difficult to manipulate.**
  2) **We report the development of new methodologies and tools to manipulate the emerging "work horse" of synthetic biology, *Vibrio natriegens*.**
  3) **We show in the second half of the manuscript (Figs. 4-5) that as a tool, this platform can be a game changer when one wishes to study single effectors or the synergy between effectors under diverse environmental conditions. Since the engineered system is functional at 20-37 °C and in a wide salinity range (new Fig. 3B), it can be used to study the impact of effectors on diverse bacteria and under various conditions. This is usually not the case when studying a natural T6SS which is tightly regulated under certain environmental conditions.**
  4) **We also provide an in-depth analysis of the regulatory network that controls the various operons and the activation sequence of the *Vibrio parahaemolyticus* T6SS1. To avoid obstructing the flow of the manuscript, we ended up pushing these data to the Expanded View Figures (Figure EV2) and summarized these results in the main text (lines 125-132).**

- It is an interesting application of what we know about T6SS but everything presented here is somewhat expected and brings no new insights into how the system works or what is its role during bacterial interactions.

  As the reviewer notes, this report describes using our accumulated knowledge on this T6SS and applying it to engineer a proof-of-concept platform. However, we ask the reviewer to also consider other aspects of this work, such as the tools that were developed, the potential use of the platform to study effectors and not only as a potential bio-treatment, as well as the in-depth analysis of the regulatory network of the T6SS in question. We provide methods that will allow small research groups to study complicated biological systems. Although this work may appear trivial once it is presented in a manuscript, it was not. Now, we believe others can follow our footsteps and study T6SSs (or other secretion systems) and effectors even from difficult to handle bacterial strain, without having to be a "big lab" with an endless pool of postdocs. We believe that we provide here a guide to make the study of secretion systems more feasible (this is now mentioned in the conclusion paragraph of the Discussion section, lines 332-337).

- I think that the potential for using the developed strain to learn more about uncharacterized T6SS effectors is not clearly shown by the authors

  We demonstrate in Figures 4 and 5 how the platform can be used to study single or multiple effectors against various prey. Our results demonstrate how effectors affect prey differently, opening a door for a system that allows many interesting questions to be answered. Therefore, we provide evidence of how the platform can be used to dissect effector activities in the future. It is important to note that in this work, we report a proof-of-concept system and demonstrate its potential use as a tool to study effectors. We believe that characterizing the activities of specific effectors is beyond the scope of this report.

- I would expect to see this type of manuscript in a specialized biotechnological journal

  As we mentioned above, there are various aspects in this work that should appeal to a basic microbiology audience and not just a biotechnological audience, such as the ability to use this tool to study individual and multiple effectors of the T6SS under diverse conditions and against diverse prey strains, as well as the dissection of the T6SS1 regulatory cascade. Therefore, we believe that the impact of this work is wide and should be published in a journal with a broad and diverse readership.

- I would say that the Figure 1 clearly shows that the T6SS in V. natriegens is expressed less and is less active than in Vibrio parahaemolyticus. This means that there is a room for major improvement of the system by more engineering, tweaking the expression level of the system

  Indeed, there is room for improvement of this platform before it can actually be used as a bio-treatment. However, the results in Figure 1 show the first step in the process. It shows the native system that was transferred into a new host that lacks the required regulation network to properly control its activation (as we show later with TfoY). In Figure 2, we show that inducible control of the system by the on/off switch dramatically improves the activity of the system (from a drop of ~1.5 order of magnitude in prey viability in panel B, to almost 3 orders of magnitude drop in panel E; lines 150-153). We would also like to point out that this work reports a proof-of-concept platform, and we acknowledge that the system is not yet fully optimized. We also elaborate on how we envision it being improved in the future in the Discussion section (lines 260-286).

- Unfortunately Figure 4 also highlights how difficult it will be to use this approach to eliminate certain bacteria from a complex mixture. The attackers had to be present in a ten-fold excess to achieve detectable decrease in abundance of certain species. This is of course also dependent on the sensitivity of the target cells and thus could be potentially improved by a careful selection of more potent toxins. Nevertheless, this experiment shows that the major limitation of using T6SS to kill bacteria is that the attacker and the prey cells have to be in a close contact and thus the attacker has be present in a significant amount to increase the chance that the attacker meets and kills its prey.

  We report here a proof-of-concept platform that indeed has various limitations, at least in its current form. Nevertheless, we acknowledge these limitations and we

delineate how possible combinations with other recently developed technologies can bypass them (e.g., the PICs reported by Ting et al, Cell Host Microbe, 2020, which also were used at similar high attacker:prey ratios to demonstrate efficiency). We predict that combining this platform with other methods that provide target-specific toxicity and attachment will permit lowering the ratio of these cells. We also note that the ration of attacker-to-prey in the experiment shown in Figure 4 is not actually 10:1, since for some effectors tested all the strains behaved as prey (and thus the actual ratio was 2:1).

As a general comment, we respectfully ask the reviewer to bear in mind that this is not a report on a ready-to-use treatment, but on rather a step forward in that direction. We set out to address certain specific limitation of using T6SS as potential treatment (as delineated in lines 81-84); we did not aim to provide a working end-product at this stage. As the reviewer noted, there is still significant room for improvement. We also wish to emphasize the importance we see in the fact that this report can also provide readers with tools and a roadmap to engineer easy-to-use bacteria, such a *Vibrio natriegens*, to study complex secretion systems that may be found in bacteria that are not amenable to genetic manipulations, that are difficult to grow, or for which the conditions that activate the secretion system remain unknown.

Referee #2

- Given the high quality of this work and its importance for the field, I strongly recommend that this paper be accepted for publication

  We thank the reviewer for acknowledging the value of our work.

- To measure T6SS-mediated killing, the authors conduct competition assays using an excess of attackers (4:1 attacker:prey, or 10:1 vs prey cultures). This is common practice and fine in itself - I understand the need to create strong killing effects for detection by the (low precision) CFU counting method. However, I feel that the authors should acknowledge that the competition conditions are somewhat idealised in favour of the attacker, and may not be representative of competition conditions in nature

  This is correct. We added a comment on this in the Discussion section (lines 292-296). Nevertheless, it is worth noting that in the 10:1 ratio competition assays, the actual ratio can also be seen as 2:1, if all potential prey (5 different strains) are summed.

- T6SS effectiveness is famously poor in liquid culture, as cell-cell contact is transient. How will this impact the effectiveness of the V. natriegens platform as a biocontrol agent in aquaculture systems? I am not sure to what extent competition will be surficial in this context; could the authors clarify? It might also be worth pointing out that the nanobody adhesion system of Ting et al. makes T6SS killing effective in liquid culture, so - in combination with this work - this could be a good way of extending the conditions in which V. natriegens is an effective killer (as well as improving specificity, as noted on L323).

  Our vision is that this platform will be used inside marine animals (e.g., in the gut

of fish or crustaceans). In this scenario, direct contact between bacteria that employ T6SS competitions have been well documented in both animals and humans. As the reviewer suggested, we now note in the Discussion (lines 273-274) the possibility of bio-treatment in liquid media conditions upon integration of our platform with the one developed by Ting et al.

- L56: I think 'lucrative' is misleading here since (notwithstanding the authors' patent application) this system has yet to be commercialised. I suggest 'promising' as an alternative.

  As the reviewer suggested, we changed 'lucrative' to 'promising' (line 63).

- L144 and Fig 2B: I do not understand why you label the vp1407 knockout pT6SS1Ind. To me, 'Ind' suggests induction, but surely the point here is that there's no induction because a key positive regulator has been removed. Maybe pT6SS1Δvp1407 would be more intuitive?

  The notion is that this mutated cluster became inducible (hence, "Ind"). If the reviewer/editor feels that this is an important point, we can change the name.

- Fig 2C: I got confused comparing this to Fig 1B. In 1B, you use asterisks to point out additional non-specific bands that appear in V. natriegens samples, which don't correspond to VgrG1. Are these bands present in Fig. 2C (and if not, why not)? I initially interpreted 2C as indicating no T6 secretion occurred under any conditions, but in fact you're showing that firing only occurs if i) there's a functional T6SS present, and ii) the arabinose inducer is provided. Is that correct? Overall, I think you just need to clarify why the extra, non-VgrG1 bands present in Fig. 1B disappear in Fig. 2C.

  We noticed that the non-specific band in question does not always appear. We speculate that it could be related to the number of cycles in which a primary antibody tube had been in use. We also note that in Fig. 2C, we show the activity of the inducible system, which is considerably more active than the unmodified T6SS cluster shown in Fig. 1B. Therefore, less exposure to see the VgrG bands, and that could also explain why the non-specific bands were not apparent.

- Fig 5C: The text and cell colours are very faint; making both larger and using bolder colours would improve readability here.

  The panel was modified as suggested.

- L 328: What is the rationale for having the exogenous effector/immunity genes controlled by the same regulator as the T6SS core? Is this a cost-reduction measure?

  The rationale is that one will only need to replace one component in the system, namely the regulation of the on/off switch, rather than the regulation of the on/off switch and that of the effector/immunity pairs. It should be more efficient and less time consuming for customizing the system. We now mention this in the text (lines 276-277).

- L349. The ability to study single effectors in isolation makes sense as an application of your system. However, comparing this with exogenous toxin expression in prey cells seems like a bit of a straw man - does anyone actually use this to assess potency?

  This is exactly the point that we are trying to make. Over-expression of effectors in *E. coli* may result in non-specific toxicity that is not always achieved by the natural T6SS-mediated competition against certain prey strains. Taking as an example the works of Prof. Tao Dong's group on TseH, the natural resistance of certain strains to this effector when delivered via the T6SS would have been missed if it was only studied upon over-expression in *E. coli*. Using our system, which is closer to natural T6SS-mediated delivery of the effectors into prey (especially effectors that act in the prey periplasm and therefore must be fused to a signal peptide or TAT secretion peptide when exogenously over-expressed in *E. coli*), one can mimic a more natural scenario (and amount) of effector delivery.

- L349. Relating to the above: surely the key advantage of your platform (over, say, just knocking out all but one effector in a T6SS+ bacterium) is that you can compare effectors from lots of different species in a single platform, where T6SS and effector expression are tightly (and externally) controlled? This seems like a great way of removing confounding factors (e.g. species A making more effector / firing faster than species B) from effector comparisons. I think it would be worth pointing this advantage out.

  We thank the reviewer for this suggestion. However, this may not always be true since we cannot really account for differences in expression levels, stability, and efficiency of loading onto the tail tube in our platform. Therefore, we believe it should be used less for comparing effectors, but rather to provide the ability to study effectors from diverse strains, as noted by the reviewer.

1st Revision - Editorial Decision 18th Aug 2021

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 your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests:

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV figures), and that statistical testing has been done where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. If statistical testing was done but there is no significant difference, please also mark this in the diagrams (n.s.).

- As they are cropped, could you provide the source data for the few Western blots shown in the manuscript (including the EV figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. It seems you already addressed these, but please re-check. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

I want to congratulate the authors on a very nice manuscript! I have no specific comments to the revised version.

Referee #2:

1) Summary

a. Does this manuscript report a single key finding? YES
If YES, please describe it in one sentence.

This paper describes the development of a customisable, proof-of-principle biotherapeutic, based on arming a host-safe strain of Vibrio natriegens with an antibacterial Type 6 Secretion System (T6SS).

As related previously, the authors demonstrate multiple significant advances towards the goal of harnessing T6SS-armed bacteria as biocontrol agents or biotherapeutics, showing an engineered V. natriegens strain with:
- Externally-controlled T6SS activity;
- Flexible T6SS effector payload, which allows antibacterial spectrum to be adjusted;
- Modular genetic architecture, such that regulatory and payload components can be freely interchanged.

Apart from immediate application as a marine biocontrol agent, the engineered strain could serve as the foundation for future antibacterial therapies, or as a platform with which to compare antimicrobial toxins. Overall, this is a major step forward towards the development of safe, effective biotherapeutics based on the T6SS.

b. Is the reported work of significance? YES

c. Is it of general interest to the molecular biology community? YES
If YES, please say why, in a single sentence.

I deem this work to be of significant general interest to the molecular biology community, given the current AMR crisis and pressing need to develop alternatives to conventional antibiotics.

d. Is the single major finding robustly documented using independent lines of experimental evidence? YES

Please see my previous review for technical assessment.

2) Suitability for publication

Suitable for publication without revision.

Given the high quality of this work and its importance for the field, I previously recommended that this report be accepted for publication. I emphatically repeat that recommendation here. I am happy that the authors have further improved their manuscript by addressing referees' criticisms and suggestions - these have done much to clarify the study's message and scope.

3) Remarks to the Author.

Thank you for your thoughtful responses to my previous comments.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 20th Aug 2021

Dr. Dor Salomon
Tel Aviv University
Clinical Microbiology and Immunology
Ramat Aviv
Sackler Faculty of Medicine
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Corresponding Author Name: Dor Salomon
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2021-53681-T

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
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	No

USEFUL LINKS FOR COMPLETING THIS FORM

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

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
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http://www.consort-statement.org
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http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

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).	anti-VgrG1 (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

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA