EMBO reports

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A Salmonella Typhi homolog of bacteriophage muramidases controls Typhoid toxin secretion

Helene Hodak and Jorge E. Galan

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Transaction Report:

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

1st Editorial Decision 18 September 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed reports on it.

As you will see, the referees agree on the potential interest of the findings. However referee #1 raises one major concern that will be crucial to address in order to make the manuscript suitable for publication. He/she is concerned that the observed toxin release might not constitute a true secretion system, but might rather occur through an unspecific process. In addition, referee #2 raises concerns regarding the issue of specificity of toxin release. Referee #2 also states that further insights in the retention mechanism of the toxin under conditions of lysis of the cell wall would strengthen the study and that the functionality of the TtsA:SEN1395 chimeras should be clarified. Both reviewers also point out a number of other issues that would need to be addressed before the study becomes suitable for publication here.

Given the potential interest of the findings and considering that both referees provide constructive suggestions on how to move the study forward, I would like to give you the opportunity to revise the manuscript, with the understanding that the main referees concerns have to be addressed and that acceptance of the manuscript would entail a second round of review. I would like to point out that it is EMBO reports policy to allow a single round of revision and thus, acceptance or rejection of the manuscript will depend on the outcome of the next final round of peer-review.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 30,000 characters (including spaces). Should you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1
This manuscript reports the interesting discovery that S. enterica serovar Typhi delivers the A/B typhoid toxin across the outer membrane by a mechanism that is dependent on production of a genetically-linked muramidase. Overall, the study is well-developed, and the authors have addressed a number of interesting questions pertaining to the role of the TtsA muramidase in secretion. One important issue that still needs to be resolved- and will determine the suitability of this work for publication - is whether the observed phenomenon is a true secretion system - as the authors claim - or a nonspecific release mechanism. The authors do not, for example, assay for TtsA-mediated release of other periplasmic or membrane-associated proteins along with the toxin. This issue is important to address, as there are already-described systems that mediate the nonspecific release of cellular contents, e.g., through cell lysis, localized permeabolization of the outer membrane, or release of membrane blebs. To claim discovery of a 'novel' secretion mechanism, the authors need to rule out other obvious - or not so obvious - possibilities.

The manuscript also needs to be tightened up - it's too verbose and redundant.

Specific comments:

1. Pg. 5, middle (line numbers would have been appreciated). The conclusion that TtsA is required for toxin secretion is premature here - other mechanisms can be envisioned, e.g., TtsA production results in outer membrane permeability.

2. Pg. 5. Bottom. The authors are confusing gene expression with steady-state protein accumulation. The westerns show steady-state accumulation, which might not accurately reflect gene expression. What about analyzing transcript levels? Minimally, the writing needs to be corrected to reflect what is actually being assayed.

3. Pg. 5 middle. Seems worth noting that Sty1887 codes for a phage-tail-like protein - even though the mutation didn't have an obvious effect on toxin release. The observation at least bolsters the proposal that TtsA was adapted from a phage system for a novel purpose.

4. Pg. 7 first paragraph - This line of study seems to support the idea that TtsA (together with an unspecified holin) functions by lysing the cells - or at least partially lysing them or a subpopulation of them. How can the authors rule out this as an alternative explanation to their proposed dedicated secretion mechanism? Maybe this is a case of bistability - a subpopulation of cells in the vacuole lyses and releases the toxin, while other cells that don't release the toxin remain intact?

5. Pg. 7/8. The earlier paper reporting this new family of hydrolases by Rothman-Denes showed a zymogram - why can't this be done here with purified TtsA? Seems to be a more direct way of

showing catalytic activity.

6. Pg. 8. Top. Last sentence - it's required for toxin release - not necessarily secretion.

7. Pg. 9. The phylogenetic analysis interesting, although again caution needs to be taken in concluding that these muramidases function in secretion vs some other type of release system(s). Maybe they mediate the release of membrane blebs that carry toxins or other cargo?

8. Pg. 11. Bottom. Would structural modeling of this domain (with phyre or equivalent) reveal anything of interest about the position of the critical Asn residue?

Referee #2
SUMMARY
In this work, Hodak and Galan identified a murein hydrolase (ttsA) encoded adjacent to the typhoid toxin gene. The authors demonstrate that ttsA is required for release of toxin from the periplasm of the host cell. Access to the peptidoglycan layer is proposed to be controlled by an unknown holin.

TtsA is shown to belong to the glycosyl hydrolase family 108 and the activity of its catalytic E residue is confirmed through SDM and complementation. Additionally, comparison with other closely related muramidases identified a conserved N residue in the C-terminal PG binding domain that was critical for typhoid toxin release, as demonstrated by domain swapping and site-directed mutagenesis experiments.

The model presented states that a holin is required for entry of the muramidase into the periplasm, where its lytic activity results in the release of typhoid toxin, however, the rationale for specificity for typhoid toxin is not clear to this reviewer.

All-in-all, the experiments are well thought-out and well controlled.

MAJOR COMMENTS
The conclusions in this manuscript are for the most part supported by the data presented; however, the manuscript could be improved by addressing the following concerns:
1. The authors state that overexpression of active PG amidases such as Sen1395 results in active PG hydrolysis (as measured by cell lysis after $CHCl_3$ exposure), but that release of typhoid toxin is not complemented. How can it be that these enzymes are creating holes in the PG layer without permitting release of the toxin? What other factors are causing its retention? Is there a direct interaction between the typhoid toxin and TtsA?
2. Did overexpression of all of the TtsA:SEN1395 chimeras result in lysis? In other words, are they all functional?

MINOR COMMENTS
1. Would it not have been simpler/cleaner to have added a signal peptide to ttsA rather than "permeabilising" the inner membrane with chloroform?
2. Figure 4F could use improvement - the tiny scissors are not so clear, maybe a break in the PG lattice would help.
3. The legend on Fig. 2E is unclear.
4. At least one of the supplementary trees could be included in the text as an entire section of the manuscript is dedicated to it.
5. Need to find the holin!

Answer to the reviewers' comments:

Referee #1

This manuscript reports the interesting discovery that S. enterica serovar Typhi delivers the A/B typhoid toxin across the outer membrane by a mechanism that is dependent on production of a genetically-linked muramidase. Overall, the study is well-developed, and the authors have addressed a number of interesting questions pertaining to the role of the TtsA muramidase in secretion.

We thank the reviewer for the positive reception of our work

One important issue that still needs to be resolved- and will determine the suitability of this work for publication - is whether the observed phenomenon is a true secretion system - as the authors claim - or a nonspecific release mechanism. The authors do not, for example, assay for TtsA-mediated release of other periplasmic or membrane-associated proteins along with the toxin. This issue is important to address, as there are already-described systems that mediate the nonspecific release of cellular contents, e.g., through cell lysis, localized permeabolization of the outer membrane, or release of
membrane blebs. To claim discovery of a 'novel' secretion mechanism, the authors need to rule out other obvious - or not so obvious - possibilities.

We are aware of systems alluded by the reviewer involving bacterial lysis as a mechanism of toxin release. However, we ruled out this possibility early on during our studies. Unfortunately, we omitted these data from our original submission. As suggested by the reviewer, we have shown in results now included in the revised version of our manuscript, that a periplasmic protein such as MalE, is not released during typhoid toxin secretion. We believe that our data show that typhoid toxin is released from the bacteria by a specific mechanism dependent on TtsA and that this mechanism does not involve bacterial lysis or non-specific membrane leakage. We hope we have addressed the reviewer's valid concerns.

The manuscript also needs to be tightened up - it's too verbose and redundant.

We have gone through the manuscript and made editorial changes to tighten it up. We would like to point out, however, that we have chosen the format of presenting the Results and the Discussion of the results together since we believe this format is more appropriate for the description of these findings. Perhaps the reviewer did not catch the fact that we use this specific format and therefore the manuscript may have come across more verbose that it really may have been. In any case, we hope that the flow of the paper has improved.

Specific comments:

1. Pg. 5, middle (line numbers would have been appreciated). The conclusion that TtsA is required for toxin secretion is premature here - other mechanisms can be envisioned, e.g., TtsA production results in outer membrane permeability.

As discussed above, we believe that the observation that a periplasmic protein is not released during typhoid toxin secretion provides more evidence for the existence of a specific mechanism of secretion/release of typhoid toxin. We agree that at this point we do not understand mechanistically how it works, something to be sorted out in the future. However, we do believe we have presented sufficient evidence that TtsA is required for the presence of typhoid toxin on the bacterial surface and the extracellular environment in a specific manner. It may be a bit semantic whether we can refer to the TtsA-dependent system as a "secretion" or "release" system. We favor "secretion" since "release" could imply that TtsA is mediating the release of the toxin from the surface, something we know is not the case since in the absence of TtsA we do not detect typhoid toxin on the bacterial surface.

2. Pg. 5. Bottom. The authors are confusing gene expression with steady-state protein accumulation. The westerns show steady-state accumulation, which might not accurately reflect gene expression. What about analyzing transcript levels? Minimally, the writing needs to be corrected to reflect what is actually being assayed.

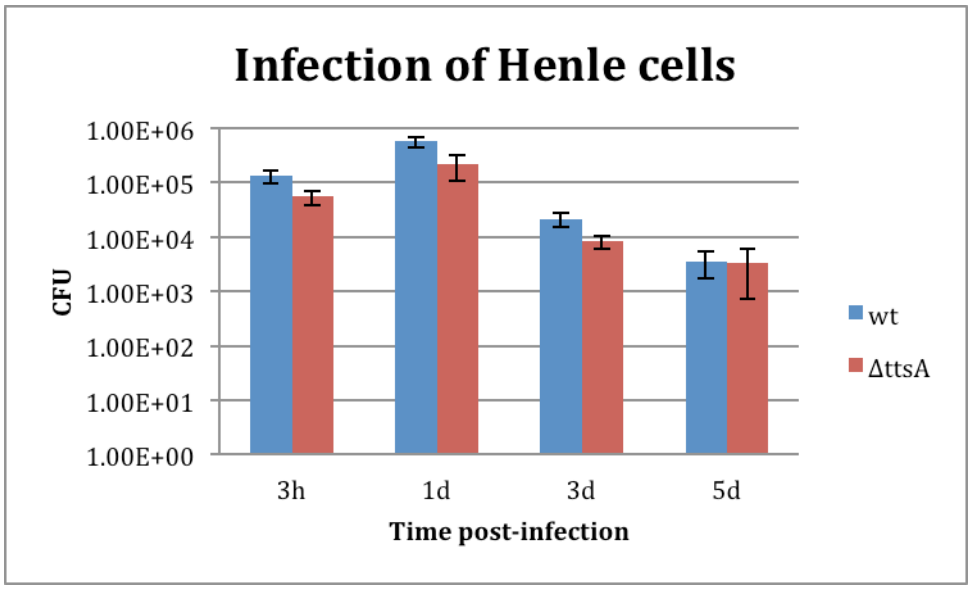
As suggested by the reviewer we have made the editorial changes to more accurately describe our findings.

3. Pg. 5 middle. Seems worth noting that Sty1887 codes for a phage-tail-like protein - even though the mutation didn't have an obvious effect on toxin release. The observation at least bolsters the proposal that TtsA was adapted from a phage system for a novel purpose.

We did notice this similarity and we agree with the reviewer that it is definitely worth pointing it out. We thank the reviewer for the suggestion.

4. Pg. 7 first paragraph - This line of study seems to support the idea that TtsA (together with an unspecified holin) functions by lysing the cells - or at least partially lysing them or a subpopulation of them. How can the authors rule out this as an alternative explanation to their proposed dedicated secretion mechanism? Maybe this is a case of bistability - a subpopulation of cells in the vacuole lyses and releases the toxin, while other cells that don't release the toxin remain intact?

The reviewer points out an intriguing alternative explanation for our results. However, we do not believe that toxin secretion is the result of the lysis of a subgroup of bacteria since a) essentially all internalized bacteria eventually release toxin, and b) there is no morphological indication that such a significant portion of the bacteria are lysing; and c) we observed no drop in cfu, which would be expected if such a large proportion of the bacterial population would be lysing (see graph included below). We have clarified this issue in the text.



5. Pg. 7/8. The earlier paper reporting this new family of hydrolases by Rothman-Denes showed a zymogram - why can't this be done here with purified TtsA? Seems to be a more direct way of showing catalytic activity.

Although Rothman-Denes group succeeded in showing catalytic activity of a bacteriophage homolog of TtsA using a zymogram-based assay, they actually reported in the paper as "data not shown" that they failed to detect muramidase activity with the same assay in Sty0016, a close homolog of TtsA encoded elsewhere in the S. Typhi. Like TtsA, Sty0016 is not predicted to be associated with phage functions since no phage genes are encoded in its vicinity. Instead, Sty0016 is encoded next to a gene encoding an a putative secreted chitinase. We believe that there is something about the activity of some of the members of this enzyme family that makes them unsuitable to work under the conditions used during the zymogram assay. Perhaps their adaptation

for functions other than phage release altered its catalytic mechanism in such a way that results in our (and Rothman-Denes group's) inability to detect its catalytic activity by the zymogram assay.

6. Pg. 8. Top. Last sentence - it's required for toxin release - not necessarily secretion.

Although as discussed above we favor "secretion" over "release", we have altered the text to accommodate the reviewers suggestion.

7. Pg. 9. The phylogenetic analysis interesting, although again caution needs to be taken in concluding that these muramidases function in secretion vs some other type of release system(s). Maybe they mediate the release of membrane blebs that carry toxins or other cargo?

We agree that the phylogenetic grouping needs to be taken with caution. We tried to convey this idea in the text of the manuscript.

8. Pg. 11. Bottom. Would structural modeling of this domain (with phyre or equivalent) reveal anything of interest about the position of the critical Asn residue?

We have modeled the carboxyterminal domain of TtsA revealing that the Asn residue is located in the middle of a loop bounded by two helixes. Although intriguing, at this point it is premature to speculate about the potential significance of this location.

Referee #2

SUMMARY
In this work, Hodak and Galan identified a murein hydrolase (ttsA) encoded adjacent to the typhoid toxin gene. The authors demonstrate that ttsA is required for release of toxin from the periplasm of the host cell. Access to the peptidoglycan layer is proposed to be controlled by an unknown holin.
TtsA is shown to belong to the glycosyl hydrolase family 108 and the activity of its catalytic E residue is confirmed through SDM and complementation. Additionally, comparison with other closely related muramidases identified a conserved N residue in the C-terminal PG binding domain that was critical for typhoid toxin release, as demonstrated by domain swapping and site-directed mutagenesis experiments.
The model presented states that a holin is required for entry of the muramidase into the periplasm, where its lytic activity results in the release of typhoid toxin, however, the rationale for specificity for typhoid toxin is not clear to this reviewer.
All-in-all, the experiments are well thought-out and well controlled.

MAJOR COMMENTS

The conclusions in this manuscript are for the most part supported by the data presented; however, the manuscript could be improved by addressing the following concerns:

1. The authors state that overexpression of active PG amidases such as Sen1395 results in active PG hydrolysis (as measured by cell lysis after CHCl3 exposure), but that release of typhoid toxin is not complemented. How can it be that these enzymes are creating holes in the PG layer without permitting release of the toxin? What other factors are causing its retention? Is there a direct interaction between the typhoid toxin and TtsA?

We used the CHCl3 treatment after TtsA overexpression as well as TtsA co-expressed with an "heterologous" holin (both resulting in bacterial lysis), as assays to demonstrate that, as predicted by its primary amino acid sequence similarities, TtsA possesses amidase activity. However, we would like to emphasize that we did not intend to imply that under normal physiological context TtsA exerts its Typhoid toxin secretion function in a similar fashion since under physiological conditions (i. e. infection of cultured mammalian cells) we do not see bacterial lysis. We do not believe it is relevant to assay for typhoid toxin release when we induce bacterial lysis because in this case obviously all cytoplasmic proteins would be released since bacteria are literally lysing. We of course believe that, under physiological conditions (i. e. during typhoid toxin release) the TtsA amidase activity must be spatially constrained, perhaps but its specific holin or other factors yet to

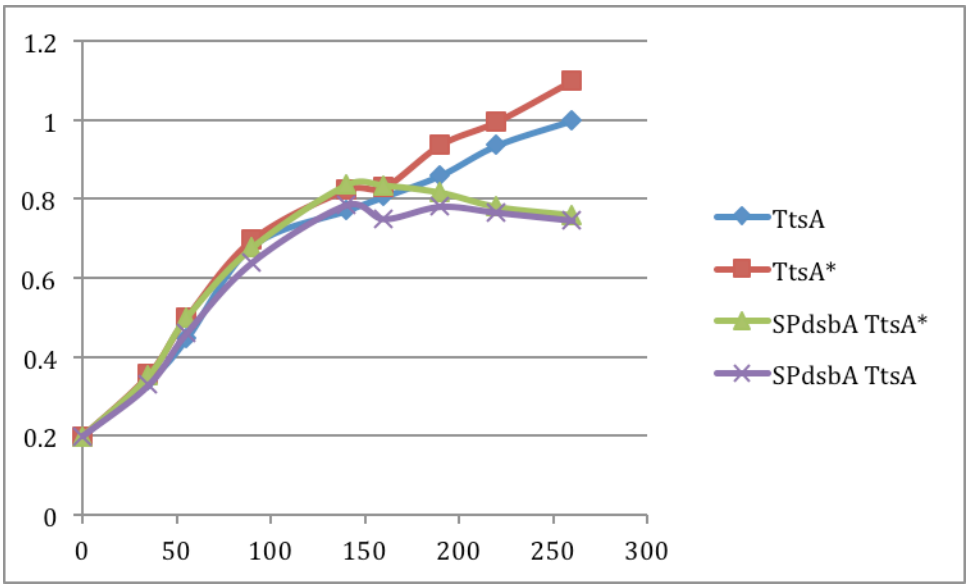
be identified. We have tried to clarify this point in the text.

2. Did overexpression of all of the TtsA:SEN1395 chimeras result in lysis? In other words, are they all functional?

Indeed, overexpression of all the chimeras resulted in bacterial lysis after CHCL3 treatment, indicating that they are "functional". We have included these data in the Supplementary Materials.

MINOR COMMENTS
1. Would it not have been simpler/cleaner to have added a signal peptide to ttsA rather than "permeabilising" the inner membrane with chloroform?

We did try experiments in which we added a signal peptide to *ttsA* (see below). However, the results were not as convincing (i. e. differences between catalytic mutant and wild type were not apparent) as those obtained with the CHCL3 treatment and co-expression with an heterologous holin. Perhaps we did not manage to get the appropriate level of expression so the we could resolve differences between the catalytic and wild type versions of TtsA due to overexpression. In any case, we did not pursue this further since the other assays worked well.



StsA*: denotes catalytic mutant
SpdsBA: denotes the presence of a signal peptide from dsbA

2. Figure 4F could use improvement - the tiny scissors are not so clear, maybe a break in the PG lattice would help.

The reviewer is correct and the model as depicted was confusing. We have altered as suggested by the reviewer.

3. The legend on Fig. 2E is unclear.

We have edited the figure legend for more clarity.

4. At least one of the supplementary trees could be included in the text as an entire section of the manuscript is dedicated to it.

We agree with the reviewer and we have now included as a main figure the graph depicting loci in which toxins or extracellular enzymes occur next to amidase/holing pair. We believe that this information is important because it gives support to our conclusion that the system describe here is not an odd occurrence in S. typhi but that is likely to constitute a more general mechanism of toxin/extracelluar enzyme release.

5. Need to find the holin!

We agree with the reviewer that finding the holin should be top priority. As the reviewer most likely knows, holins are not conserved so there are not easy to spot bioinformatically. Consequently, we have initiated a genetic screen to identify it but certainly this is beyond the scope of this manuscript.

2nd Editorial Decision 12 October 2012

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, both referees are now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor corrections have been addressed by adding the following comments in the discussion, as suggested by referee #2:

1) Include a statement that the specificity of the C-terminus may lead to the creation of openings in the PG layer that are proximal to the pool of typhoid toxin within the periplasm
2) Add a sentence about the periplasmic expression studies and the observed lack of phenotype for the activity mutant
3) If space allows it, move Supplementary figure 12 into the body of the manuscript

I have also noted that the length of the revised manuscript is currently ~40,000 characters, when it should not exceed 30,000 characters (including title page, abstract, references, figure legends and spaces, but excluding tables and Supplementary Information) . I would suggest that to reduce the character count, you move some of the Materials and Methods section to Supplementary Material, bearing in mind that the essential information for interpretation of the results should remain in the Materials and Methods.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1

This modified manuscript has satisfactorily all of my prior comments, and I believe, those of the other reviewer. I have no further revisions to request of the authors for this well-developed and thought-provoking study

Referee #2

The revisions to EMBOR-2012-36494V1 have sufficiently addressed major and minor concerns. The only suggestion I have to further improve the manuscript is to address the same issues that I initially brought up by adding a few statements in the discussion: a statement that the specificity of

the C-terminus may lead to the creation of openings in the PG layer that are proximal to the pool of typhoid toxin within the periplasm would add to the proposed model and explain why heterologous muramidases cannot complement toxin secretion. Also, a sentence about the periplasmic expression studies and the observed lack of phenotype for the activity mutant may prove useful for others working with this class of enzyme. I would also like to see Supplementary figure 12 moved into the body of the work as its findings feature highly in the discussion.

2nd Revision - authors' response 15 October 2012

We are delighted that our paper is in principle suitable for publication. We have made all the indicated changes and shortened the manuscript, which is now well within the guidelines. However, we have retained Fig. S12 as supplementary figure due to space constrains. We hope the manuscript is now ready for publication. Thank you very much for the handling our submission.

3rd Editorial Decision 25 October 2012

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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,

Editor
EMBO reports

