

Manuscript EMBO-2016-94024

TssA forms a gp6-like ring attached to the type VI secretion sheath

Sara Planamente, Osman Salih, Eleni Manoli, David Albesa-Jové, Paul Freemont and Alain Filloux

Corresponding author: Alain Filloux, Imperial College London

Review timeline:

Submission date:	3 February 2016
Editorial Decision:	15 February 2016
Revision Received:	06 April 2016
Editorial Decision:	13 May 2016
Revision received:	20 May 2016
Accepted:	23 May 2016

Editor: David del Alamo

Transaction Report:

Please note that the manuscript was transferred from another journal where it was originally reviewed. Since the original reviews are not subject to EMBO's transparent review process policy, the reports and author response cannot be published.

(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

15 February 2016

Thank you for the submission of your manuscript entitled "A gp6 ring-like structure at the tip of the type VI secretion sheath" and please accept my apologies for the delay in getting back to you. We have now received the comments from the arbitrating referee, which I copy below.

As you can see from his/her comments, the referee is rather supportive of your work although s/he believes that to some extent the conclusions are not fully supported by the evidence presented and need to be toned down. S/he also points out to a number of concerns that will require your attention before your manuscript can be published in The EMBO Journal. I will not repeat here these issues, which I believe are rather clear, but please notice that they may require additional experimental evidence in at least some cases.

Arbitrating Referee Report

I think the answer is that the paper could be interesting IF they will do more to address the referees' concerns. In general the figures are relatively poor and could do with a lot of tidying.

In general I think they need to be willing to tone down the language in the paper to acknowledge the fact that their data may suggest lots of things but don't really provide conclusive evidence for much at this point in time... additionally the experiments the referees suggest that I think are reasonable for them to do better are:

1. Mass Determination - Ref#1 points out that they seem to have used the wrong AUC experiment for shape

independent mass determination. They do not address this in their response and they also give no error estimate on the mass. Their explanation for why they can't do SEC-MALS is poor - if the sample is really a mix of dodecamers and aggregates of this it should be entirely possible to find a column to separate these species and then get a mass from SEC-MALS

On a related point - their evidence that the C-terminally deleted protein is monomeric is accepted by the two referees but looks rather dodgy to my eyes - the mass estimate is sloped and suggests that this construct is aggregating (whether in a biologically relevant way or simply because the protein is unhappy cannot be distinguished). The claim that they know which portion of the protein is driving oligomerisation is therefore overstated.

2. EM - neither of the referees particularly comment on the EM but I think there are some issues with this. Again it seems rather over interpreted given the images presented (although I haven't seen the supplement where further analysis is reported). The level of agreement between the re-projection of the model and the images is suggestive but not conclusive.

3. Which end - I suspect rather than doing further fluorescence experiments it would be better just to tone down the language surrounding this.

4. Ref#4 describes the fluorescence data as not compelling - I would tend to agree and think that the authors should address these concerns if they want to present the fluorescence data in the paper.

1st Revision – author's response

15 February 2016

ANSWER TO REVIEWER'S COMMENTS

We have now prepared a revised version of our manuscript entitled “TssA forms a gp6-like ring attached to the type VI secretion sheath” which replaces the previous title “A gp6 ring-like structure at the tip of the type VI secretion sheath”.

We have carefully considered the comments of the arbitrating referee, and you will find below a point-by-point answers to these comments with our answers written in blue. The comments are mainly querying biochemical and EM approaches which are now described in more detail while some have been added or repeated.

In addition to address the referee comments, we have included in the discussion a paragraph which comments on the recent paper by Zoued et al., published in Nature where the structure and fate of a TssA protein from the Escherichia coli T6SS (EcTssA) is described (Zoued et al., Nature, 2016, 531(7592):59-63).

What is noticeable is that this particular EcTssA is remarkably different from the one we studied here (PaTssA1) and our phylogenetic analysis (added in Figure 1 of the revised version of our paper), shows that they belong to distinct subclasses. Moreover, we found that the structure of the C-terminal domain of EcTssA (PDB code 4YO5) that they report, is missing in our TssA1 but shows instead structural similarities to TagJ, an accessory component of the P. aeruginosa T6SS-H1, (Fig EV4 and EV5 in our manuscript). We also provide evidence that TagJ interacts with PaTssA1, which is in agreement with the newly identified “TagJ-like extension” found in EcTssA and absent in PaTssA1 (Fig EV4C).

Overall the discrepancy between the two studies matches very nicely with the two subclasses of T6SS that we have previously reported (Förster et al., JBC, 2014, 289:33032-33043), one using the TssA1/TagJ system whereas the other is using a TssA from the EcTssA family. Most importantly the Zoued et al. study fails to report the homology with the gp6 C terminus which is the core piece of information in our paper, together with the fact that TssK is a gp8 homologue. From this angle we are confident that our study is fully original and distinct/complementary from the Zoued et al. paper.

1. Mass Determination - Ref#1 points out that they seem to have used the wrong AUC experiment for shape independent mass determination. They do not address this in

their response and they also give no error estimate on the mass.

We believe that we used appropriate biochemical methods and give here below the main reasons.

Since the previous version was written for Nature, the details on the experimental approaches may have been too scarce to make clear what we have actually been doing which is also fixed in the new materials and methods written for the EMBO J version. In brief, sedimentation velocity AUC experiments have given accurate molecular weight values for protein complexes, including ring shaped complexes, as shown in the following peer reviewed articles:

(Okemefuna et al., 2009, J Mol Biol 91(1):119-35; Niewiarowski et al., 2010, Biochem J, 429(1):113-25; Ando et al. 2011, PNAS, 108(52):21046-51).

A notable advantage of the velocity sedimentation is that it usually requires only several hours over the sedimentation equilibrium (several days), thus sedimentation velocity can be used with samples that are unstable. To avoid problems due to the lack of the stability of TssA1 complex over the course of the experiment (carried out at 20°C) we therefore used this AUC method to estimate the molar mass of TssA1 in solution.

A detailed description of the AUC experiments performed in this study is provided below.

Two independent preparations of the purified TssA1 have been analysed by sedimentation velocity experiments at different rotor speed (20,000 rpm and 30,000 rpm).

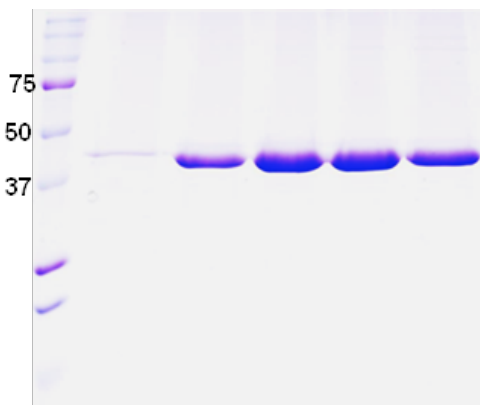


Figure A. SDS-PAGE analysis of TssA1 after purification steps

The purity of the sample is shown by the SDS-PAGE in Figure A.

The sedimentation coefficient distribution function $c(s)$ was calculated by direct fitting of the sedimentation boundaries (use of SEDFIT software) for all data sets. Good fits to the sedimentation boundaries were obtained in all cases and a representative fit is shown in Figure 2C of our new version. One of the parameters used to optimize the $c(s)$ fit, is the frictional ratio (f/f_0) from which shape information can be inferred. During $c(s)$ fit optimization the frictional ratio has been floated till the RMSD values and the fits were suitable. The error estimate on the mass of the peaks observed in all the size-distribution analyses $c(s)$ is provided in the revised version of the manuscript.

Their explanation for why they can't do SEC-MALS is poor - if the sample is really a mix of dodecamers and aggregates of this it should be entirely possible to find a column to separate these species and then get a mass from SEC-MALS.

We agree with the concern of the arbitrating referee. In an attempt to separate TssA1 complexes from the aggregates, an optimal SEC column (the WYATT 100S5 column with 50,000–7,500,000 Da separation range) has been used coupled to a MALS instrument in order to get a good separation of the TssA1 complexes from aggregates. Adoption of this new condition allowed separation of TssA1 complexes from large aggregates as shown in Figure EV1A of the revised version. The SEC profile indicates TssA1 is polydisperse in solution.

SEC-MALS data indicate that the first eluted peak (elution volume of about 10 ml) has a molar mass of 1.0 ± 0.1 MDa which is in agreement with the AUC data where a minor peak at 28.3–29 S with mass estimate of 1.05 ± 0.05 MDa is also observed. The SEC profile shows additional peaks between elution volume range 12 to 14 ml, unfortunately, due to peak overlap, the accurate molar masses of these peaks could not be assessed. In conclusion, AUC remains the most reliable technique to estimate the molecular mass of TssA1 in solution as it allowed detection of the different species present in the sample from the peaks in the size distribution analyses $c(s)$. Moreover, sedimentation velocity experiments permit distinction of the most abundant TssA1 species present in solution, indicating that TssA1 is mainly a dodecamer.

On a related point - their evidence that the C-terminally deleted protein is monomeric is accepted by the two referees but looks rather dodgy to my eyes - the mass estimate is sloped and suggests that this construct is aggregating (whether in a biologically relevant way or simply because the protein is unhappy cannot be distinguished). The claim that they know which portion of the protein is driving oligomerisation is therefore overstated.

SEC-MALS experiments using the truncated TssA1₁₋₂₄₅ protein have been repeated using optimal concentration of the protein and best experimental conditions. The SEC-MALS data (Figure 3D) showed the TssA1₁₋₂₄₅ elution peak with a molar mass of 28.4 ± 1.06 kDa which correspond to a monomer (theoretical MW of the protein is about 27 kDa). The MALS plot across the UV trace of the peak does not appear sloped in the latest experiment. Additionally, AUC experiments have been carried out using the purified truncated protein and confirm the monomeric state of TssA1₁₋₂₄₅ (Figure EV1 C). SEC-MALS (Figure 3D), in vitro cross-linking and AUC (Figure EV1, panels B and C) data showed that the truncated TssA1₁₋₂₄₅ protein is a monomer which strongly suggest that the C-terminal region of TssA1 is involved in the oligomerisation.

2. EM - neither of the referees particularly comment on the EM but I think there are some issues with this. Again it seems rather over interpreted given the images presented (although I haven't seen the supplement where further analysis is reported). The level of agreement between the re-projection of the model and the images is suggestive but not conclusive.

Additional information/description of the TssA1 single-particle analysis has been added in the latest version of the manuscript (Figure 3A and B and Figure EV2 in the new version of our manuscript).

We now provide a new 2D classification carried out using RELION and 12 representative classes of the TssA1 complexes are shown in Figure 3A, right panel. This clearly shows that TssA1 complexes are distinct rings which exhibit profound conformational heterogeneity and display a variety of symmetries ranging from three-fold to six-fold like symmetry. TssA1 complexes assume preferentially top-view orientations with very few side-views. Representative classes of TssA1 sideviews are shown: the Class 7 in Figure 3A (RELION classification), Classes 5 and 10 in Figure EV2A (IMAGIC-5 classification).

Additionally, a double MSA classification of TssA1 particles has been performed and shown in Figure 3B. This analysis highlights changes in external lobe position (Figure 3B, right panel, coloured circles), which is in agreement with our hypothesis that the TssA1 ring complex is highly flexible, although it is difficult to completely eliminate in-plane rotational alignment heterogeneity.

We agree that comparisons between reprojections of gp6_C rings and EM images of TssA1 rings are ultimately suggestive rather than conclusive. However, we undeniably observe similarities in terms of shape and size between the TssA1 and gp6_C rings that are clearly demonstrated in Figure EV2 of our new version. TssA1 rings have diameter, width and central hole dimensions of ~260 Å, ~95 Å and ~100 Å, respectively, and are clearly comparable to those measured for gp6_C rings at ~240 Å, ~80 Å and ~100 Å, respectively. These observations, together with significant sequence similarity (~62 %) detected between the C-terminal regions of TssA1 and gp6, hint at structural parallels existing between these two ring complexes.

3. Which end - I suspect rather than doing further fluorescence experiments it would be better just to tone down the language surrounding this. 4. Ref#4 describes the fluorescence data as not compelling - I would tend to agree and think that the authors should address these concerns if they want to present the fluorescence data in the paper.

We agree with the referee and we have toned down the interpretation and discussion of the fluorescence microscopy data which are now kept to a minimum and simply used to show the difference in impact of TssA when compared to other T6SS components. In brief, only clear observations showing the presence of TssB1 foci being decreased in the tssA1 mutant population compare to the WT cells, are described. Hypothesis regarding sheath dynamics in the observed strains has been removed as suggested by the referee.

2nd Editorial Decision

13 May 2016

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

Browsing through the manuscript myself I have noticed a few cosmetic issues that will need to be addressed in the final version of the paper. Essentially, figures involving BTH beta-gal measurements need to be described in better detail. In particular, error bars shown in figures 2A, 4D and 7 need to be defined in the figure legend. Although also mentioned in the methods section, it would also be helpful to include information regarding number of experiments performed.

 REFEREE REPORTS

Referee #1:

The authors have added significant new data that, whilst not changing their earlier interpretations, substantially strengthen the data presented in support of their ideas. This, taken together with the textual alterations, make the manuscript suitable for publications.

2nd Revision - authors' response

20 May 2016

Many thanks for your feedback on the manuscript and we are all extremely pleased with the accept decision.

I have also followed your recommendation as for the cosmetic issues you mentioned in your letter and this has all been fixed accordingly in the revised version I have uploaded.

3rd Editorial Decision

23 May 2016

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:

Journal Submitted to:

Manuscript Number:

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

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

A- Figures**1. Data****The data shown in figures should satisfy the following conditions:**

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

2. Captions**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	In order for good statistics, a large enough number of particles were collected and analysed (~3,000) (see reslut and method ssesseions page 6 and 21 of the submitted manuscript), sufficient to identify heterogeneous states of TssA1. This particle number is comparable to that used in the statistical analysis of 2D projections by Elad et al., 2008, whose methodology was followed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Initially, no particles were excluded from single-particle analysis. Then, after each round of classification, particles sorted into visually poor looking groups (i.e. no distinct particle shape or structural features, poor contrast and/or mis-alignment), were excluded from further analysis. The exclusion criteria were pre-established as this is general, standard practice during EM single-particle image analysis.
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.	The effects of subjective bias were avoided/minimized by independently performing 2 sets of EM single-particle image-analysis, using 2 different software packages (RELION and IMAGIC-V). Both strategies gave similar class averages, providing clear evidence for the existence of heterogeneous states of TssA1 (see reslut and method ssesseions page 6-7 and 21 of the submitted manuscript).
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Please refer to box B,3 above.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	N/A
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	Standard practice for EM image processing and analysis is to normalise the data set for subsequent classification. Once normalised, classification aims to minimise intra-class variance whilst maximising inter-class variance in order to identify different groups of images that may represent a different complex orientation or a different conformational state. The variance between different classes is therefore greater than within a class, and is taken into account internally by the software during the classification procedure.

USEFUL LINKS FOR COMPLETING THIS FORM<http://www.antibodypedia.com><http://1degreebio.org><http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo><http://grants.nih.gov/grants/olaw/olaw.htm><http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm><http://ClinicalTrials.gov><http://www.consort-statement.org><http://www.consort-statement.org/checklists/view/32-consort/66-title><http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun><http://datadryad.org><http://figshare.com><http://www.ncbi.nlm.nih.gov/gap><http://www.ebi.ac.uk/ega><http://biomodels.net/><http://biomodels.net/miriam/><http://ijb.biochem.sun.ac.za>http://oba.od.nih.gov/biosecurity/biosecurity_documents.html<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	Please refer to the box above.
---	--------------------------------

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).	All the antibodies against T6SS components (anti-TssA1, anti-TssB1, anti-Hcp1, anti-tse1 and anti-VgrG1) have been raised in rabbit and obtained from Eurogentec. Monoclonal Anti-polyHistidine Clone HIS-1 (Mouse Ascites Fluid), product Number H 1029 (SIGMA); RNA pol β (NT63): sc-101596 (SANTA CRUZ BIOTECHNOLOGY); Anti-Rabbit IgG (whole molecule)-peroxidase, produced in goat, affinity isolated antibody, product Number A 6154 (SIGMA); Anti-Mouse IgG (whole molecule)-peroxidase, IgG Fraction of antiserum, product Number A 9044 (SIGMA)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	N/A
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).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: Primary Data Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4Q26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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 Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

23. 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.	N/A
---	-----