EMBO reports

High-resolution structure of native toxin A from *Clostridioides difficile*

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Jørgensen,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

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 or in the 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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http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

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, structural and array data) are deposited in an

appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

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

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

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

Moreover, I have these editorial requests:

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

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, please make sure that, where applicable, the number "n" for how many independent experiments were performed and the type of replicate (biological or technical), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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10) Please restrict the number of key words to up to 5 and the number of words for the abstract to 175 and order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods -Data availability section - Acknowledgements - Author contributions - Conflict of interest statement - References - Figure legends - Expanded View Figure legends.

Finally, please note that all corresponding and co-corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines: http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

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

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

This Cryo-EM structure provides new insight into the specific interactions in the pore-forming region which was not available in the x-ray crystal structure as the CROPs domain was missing. However, the authors use the pH 5.2 crystal structure of TcdB to propose rotation of the CPD and GTD domains in relation to the CROPs domain from neutral to low pH. This superposition of the TcdB crystal structure onto the TcdA cryo-EM structure ignores the low resolution TcdA Cryo-EM envelope published by Pruitt et al which suggests compaction of the GTD domain rather than rotation of the CROPs domain. Given the high technical skill of the authors with Cryo-EM, I would like to see a Cryo-EM structure of TcdA at low pH rather than a structural superposition onto TcdB as proof of the CROPs rotation over compaction of the GTD domain. In addition, the authors propose that their structure is low pH, yet the Cryo-EM envelope more closely matches the neutral pH Cryo-EM envelope published by Pruitt et al and while there is some discussion that this full-length TcdA structure may be a transitional state from neutral to low pH, I think the hypothesis would be better supported by a Cryo-EM structure at low pH than a superposition onto TcdB. As conditions for a low pH Cryo-EM state of TcdA have already been published, it should not be onerous to determine a high resolution structure with the advances in Cryo-EM technology since 2010.

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TcdA (~308 kDa) and homologous TcdB (270 kDa) are among the largest protein toxins and major virulence factors of C. difficile. The full-length crystal structure of TcdB was recently published, and the crystal was formed under low-pH conditions (pH5.2) with assistance of three nanobodies. The crystal structure of a truncated TcdA lacking the C-terminal CROPs domain has previously been published a few years ago and this crystal was also under low pH condition (pH6.0). A low-resolution EM image of TcdA has been published 10 years ago, showing the relative position of the large CROPs domain to the rest of the molecule. The current work reports the first TcdA structure containing the CROPs domain under neutral pH condition. This is achieved using cryo-EM approach, and the authors were able to resolve an impressive high-resolution structure at 2.8A. Such a high-resolution allowed the author to resolve several key structural details missing from all previous structures, including direct contact of CROPs to the distal tip of the translocation domain and the contact between a subdomain (GSD) and the pore-forming regions. The authors proposed that these interactions may serve to protect and stabilize the hydrophobic pore-forming segments. The author also proposed that the hinger region may be considered as a part of the CROPs and play key roles in determining the position of the CROPs. This high-resolution structure showcases the power of cryo-EM approach and represents a major advance in understanding the structure and function of TcdA and TcdB. The manuscript is very well written, and the figures are clear and well arranged.

The CROPs location in this TcdA structure is consistent with the one suggested in previous low-resolution EM images, but dramatically different from the CROPs in the reported TcdB. The authors and previous models suggest that CROPs may rotate into a different position at low pH conditions. Is it possible for the authors to image TcdA under low pH conditions using cryo-EM? This will greatly increase the impact of the work. As a minimum, any analysis of negative staining images under different pH conditions? and a brief discussion on any technical difficulties encountered by the authors.

Minor points:
Fig. 1A: change "translocation" to "translocation and receptor-binding" or use "DRBD" adopted by others.
Line 56: recent relevant references on receptors can be cited here (Yuan et al, Cell Research, 2015, LaFrance et al, PNAS, 2015, Tao et al, Nature, 2016, Tao et al, Nature Microbiology, 2019, Chen et al, Nature Communications, 2021).
Line: 57: delete this sentence "The toxins are internalized ..... early endosomes.", and "Here,".
Line 62: cite the new reference from Orrell et al, Nature Communications, 2020, 11:432.
Line 67: The reference Orrell et al, 2017 can be replaced with better choices from Klaus Aktories.
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Please specify "strictly conserved" among what? (as in Line 336)
Negative staining images of the native sample and 2D classification should be presented.

In line 133 "from residue 2 to 2383 of the complete 2710 residues constituting the full-length TcdA". Is there only one class in final model?

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In this manuscript, Aminzadeh and colleagues present a high-resolution cryoEM-structure of TcdA, a large AB-type toxin from Clostridioides difficile. TcdA is the "little brother" of the closely related TcdB, but with the length of over 2700 amino acids, it still

is an intimidatingly large protein (at least for a protein crystallographer such as this referee) that has imposed enormous challenges to the community of structural biologists before structures of the complete protein could be determined some years ago (my group and I have utterly failed ourselves some while back). With few notable exceptions for the TcdA/B toxins, where some crystal structures have been published recently, work with such large proteins has been revolutionized by the advent of new hardware for cryoEM structure determination, and the manuscript by Aminzadeh et al. sets an excellent example for this.

The authors show us a cryoEM structure of the complete TcdA toxin at neutral pH, which reveals important information about the conformation that the toxin likely adopts before it docks to the host cell and adopts a translocation-competent conformation upon acidification of the endosome that takes it to the inside of the cell. These data add nicely to previously reported structures that have been obtained at lower pH, and because of the high resolution of this new TcdA structure (2.8 A), reveal insight into details of the underlying "mechanics" of conformational changes associated with the intoxication mechanism of these toxins. Because the structure has been obtained at neutral pH, the authors can identify means of keeping the protein in a pre-translocation state and make compelling arguments about conservation of this locking mechanism by showing high sequence conservation of the respective residues across several related proteins. The manuscript describes only structural work without biochemical experiments to corroborate the analysis of the structure, but this may be ok in the light of this being a "report" and the authors referring to other literature discussing other experimental data. Altogether, the data presented here broaden our view about these important and fascinating virulence factors.

The paper is well written and contains clearly presented figures. The work is technically sound, and I have only very few comments:

- Line 53/54: the authors seem to state that Fig. 1A shows the multi-step mechanism that TcdA/B use to enter host cells, but it doesn't. It may be helpful to readers not familiar with these toxins to provide a general sketch showing this mechanism (possibly as a supporting figure).
- L82/86: I am personally not a fan of the word "solved" when it comes to talking about crystal structures - it is lab jargon referring to the step of initial phasing rather than the whole process of structure determination. I therefore prefer "determined", but I leave it up to the authors to reword the text or not.
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- L305/306: the authors state that their TcdA structure is in line with an older low-resolution structure of Pruitt et al. from 2010 - I wonder if this could be shown in a (supporting) figure.
- L333/334: the authors state that the contacts found in their new TcdA structure "prevent premature pore formation until reaching the endosomal compartment". I wonder what they envision here: I guess that if one simply lowers the pH of a solution containing these toxins, the proteins will simply precipitate due to aggregate formation after exposing hydrophobic regions without having a membrane into which these regions could be inserted. Hence, there would be no "premature pore formation". Or do they envision insertion into the plasma membrane of the cell? I guess none of us knows what these pores look like as yet - is one copy of the protein enough to establish translocation, or do several proteins have to come together? Is the translocated cargo unfolded or not?
- The manuscript evolves around comparisons with previously published structures. Without knowing details of this work, but maybe the authors can obtain even more insight by also discussing a recently published paper by Peng Chen et al. (doi: 10.1038/s41467-021-23878-3.), which shows the structure of a fragment of TcdB in complex with a receptor.

Response to reviewers

Please note that line numbers mentioned below refer to the line numbers in the attached word document with track changes as these are different from the numbers in the converted PDF file.

Referee #1: Authors' response in blue

Comment 1:

This Cryo-EM structure provides new insight into the specific interactions in the pore-forming region which was not available in the x-ray crystal structure as the CROPs domain was missing. However, the authors use the pH 5.2 crystal structure of TcdB to propose rotation of the CPD and GTD domains in relation to the CROPs domain from neutral to low pH. This superposition of the TcdB crystal structure onto the TcdA cryo-EM structure ignores the low resolution TcdA Cryo-EM envelope published by Pruitt et al which suggests compaction of the GTD domain rather than rotation of the CROPs domain. Given the high technical skill of the authors with Cryo-EM, I would like to see a Cryo-EM structure of TcdA at low pH rather than a structural superposition onto TcdB as proof of the CROPs rotation over compaction of the GTD domain.

We assume that Referee #1 refers to the low-resolution negative stain EM of TcdA determined at both neutral and low pH (Pruitt et al., 2010, PNAS doi: 10.1073/pnas.1002199107.). It is our belief that the domain organization and the GTD compaction is a misinterpreation (by the authors) of the low-resolution negative stain structures. In Pruitt et al., figure 8 (figure 1A below) [Figures for referees not shown.] the authors suggested that the head of TcdA (yellow) is the translocation/delivery domain and that this domain changes into an elongation conformation upon lowering the pH (figure 1B). In addition, the short tail (blue and red) was suggested to consist of the autoprotease (CPD) and the glycosyltransferase (GTD) domains with the latter loosing structural stability at low pH (Figure 1B).

However, when the crystal structure of TcdA without the CROPs was published in 2016 (Chumbler et al., 2016) the domain organization was shown to be different (Figure 2). It turned out that the head contains the CPD and the GTD domain while the short tail is the Delivery domain.

With that in mind, we believe that the domain organization of figure 1B in the Pruitt et al., 2010 paper is correct and that what they see is actually a rotation of the CROPs domain very similar to what is shown in the crystal structure of the highly homologous TcdB (Chen et al., 2019). An attempt to illustrate this is shown in a new figure (Fig. EV2B and C) in the revised manuscript. This figure is also in line with the later interpretation of the negative stain EM in the review from Borden Lacy's lab (Chandrasekaran & Lacy, 2017).

It should be noted that the CROPs domain of TcdA is almost 350 amino acids longer than in TcdB. However, the Pruitt et al. paper also demonstrates a conformation of TcdB at neutral pH which is similar to TcdA at neutral pH, only shorter. Therefore, considering the high structural similarity between TcdA and TcdB, we are confident that a structural superposition of the two toxins is justifiable.
Fig. EV2B and C is now included in the Expanded view section to support that TcdA and TcdB show similar structural dynamics when going from neutral to endosomal pH.

Comment 2:
In addition, the authors propose that their structure is low pH, yet the Cryo-EM envelope more closely matches the neutral pH Cryo-EM envelope published by Pruitt et al and while there is some discussion that this full-length TcdA structure may be a transitional state from neutral to low pH, I think the hypothesis would be better supported by a Cryo-EM structure at low pH than a superposition onto TcdB.

We are not sure what Referee #1 means with this comment. We propose in the manuscript that our structure is a neutral pH structure (not low pH) and that it represents a pre-receptor-binding state. This is specified throughout the text, for instance in lines; 30, 38, 131-132, 179, 205, 206, 215, 223, 227, 383, and more…

Comment 3:
As conditions for a low pH Cryo-EM state of TcdA have already been published, it should not be onerous to determine a high resolution structure with the advances in Cryo-EM technology since 2010.

We agree that determining a high-resolution cryo-EM structure of TcdA at low pH is highly desirable. Therefore, we also collected images of samples prepared in a low pH buffer during the limited time that we had access to the Titan Krios cryo-EM facility. Unfortunately, we experienced a high degree of protein precipitation and low-resolution 2D classes likely due to non-optimal sample conditions and we therefore failed to get useful images for determining a high-resolution structure at low pH. Optimization of the low pH conditions is ongoing work, and we hope to complete this work in the future. However, it is not possible for us to achieve this within the three-month deadline for the resubmission of this manuscript. Nonetheless, as mentioned in the response to comment 1, we have tried to further clarify the justification of using a superposition of the low pH TcdB structure in line 392-397, and by including a figure (Fig. EV5B and C) in the Expanded view section.

Referee #2: Authors' response in blue

Comment 1:
TcdA (~308 kDa) and homologous TcdB (270 kDa) are among the largest protein toxins and major virulence factors of C. difficile. The full-length crystal structure of TcdB was recently published, and the crystal was formed under low-pH conditions (pH5.2) with assistance of three nanobodies. The crystal structure of a truncated TcdA lacking the C-terminal CROPs domain has previously been published a few years ago and this crystal was also under low pH condition (pH6.0). A low-resolution EM image of TcdA has been published 10 years ago, showing the relative position of the large CROPs domain to the rest of the molecule. The current work reports the first TcdA structure containing the CROPs domain under neutral pH condition. This is achieved using cryo-EM approach, and the authors were able to resolve an impressive high-resolution structure at 2.8A. Such a high-resolution allowed the author to resolve several key structural details missing from all previous structures, including direct contact of CROPs to the distal tip of the translocation domain and the contact between a subdomain (GSD) and the pore-forming regions. The authors proposed that these interactions may serve to protect and stabilize the hydrophobic pore-forming segments. The author also proposed that the hinger region may be considered as a part of the CROPs and play key roles in determining the position of the CROPs. This high-resolution structure showcases the power of cryo-EM approach and represents a major advance in understanding the structure and function of TcdA and TcdB. The manuscript is very well written, and the figures are clear and well arranged.

The CROPs location in this TcdA structure is consistent with the one suggested in previous low-resolution EM images, but dramatically different from the CROPs in the reported TcdB. The authors and previous models suggest that CROPs may rotate into a different position at low pH conditions. Is it possible for the authors to image TcdA under low pH conditions using cryo-EM? This will greatly increase the impact of the work.

We agree with Referee #2 that a cryo-EM structure at low pH will increase the impact of the work and add credibility to the claimed movement of the CROPs. We collected images of samples prepared in a low pH buffer during the short time that we had access to the Titan Krios cryo-EM facility. As mentioned in the response to comment 3 from Referee #1, we experienced a high degree of protein precipitation and low resolution and failed to get useful images for determining a high-resolution structure at low pH. We expect to try again with optimized samples conditions in the near future, but this is not possible to achieve within the three-month deadline for the resubmission of this manuscript. We have included a figure (Fig. EV5B and C) in the Expanded view section, which demonstrate that TcdA and TcdB show similar structural dynamics when going from neutral to endosomal pH.

Comment 2:
As a minimum, any analysis of negative staining images under different pH conditions? and a brief discussion on any technical difficulties encountered by the authors.

Negative staining of TcdA at low and neutral pH has already been published by Pruitt et al, 2010, and we have now added a figure to the expanded view section in the manuscript (Fig. EV5B and C) describing these previous negative staining results. We have so far not succeeded with determining a high-resolution cryo-EM structure of TcdA at low pH, but we are working on optimizing sample conditions as ongoing work for a future publication.

Minor points:

Fig. 1A: change "translocation" to "translocation and receptor-binding" or use "DRBD" adopted by others.

This domain has been renamed to DRBD (Delivery and Receptor-Binding Domain) in Fig. 1A and throughout the rest of the manuscript.

Line 56: recent relevant references on receptors can be cited here (Yuan et al, Cell Research, 2015, LaFrance et al, PNAS, 2015, Tao et al, Nature, 2016, Tao et al, Nature Microbiology, 2019, Chen et al, Nature Communications, 2021).

References have been updated to the relevant ones suggested by the referee. Thank you for reminding us about these papers.

Line: 57: delete this sentence "The toxins are internalized ..... early endosomes.", and "Here,".

Sentence is deleted.

Line 62: cite the new reference from Orrell et al, Nature Communications, 2020, 11:432.

This reference has been added. Thank you.

Line 67: The reference Orrell et al, 2017 can be replaced with better choices from Klaus Aktories.

The Orrell et al. reference has been replaced by the Klaus Aktories review in Annual Review of Microbiology from 2017.

Line 112: needs more introduction on GSD and relevant references.

Short introduction on GSD (including references) has been added to the Introduction (line 66-69).

Line 234: here short repeat and long repeat needs to be introduced. Move some parts of next paragraph here (or to introduction).

Short introduction to short and long repeats added to the Introduction (line 60-61).

Line 395: this paragraph is an over-statement, delete it or replace with the focus on the structural insight.

The last part of this paragraph has been deleted and replaced with a sentence focusing on the structural insight (line 676-677).

Please specify "strictly conserved" among what? (as in Line 336)

We have specified in the text that "strictly conserved" refers to the conservation within family of LCTs. Furthermore, we have added a paragraph (line 634-641) from the recent publication by Orrell et al., 2020, which reports an evolutionary conservation of the translocation domain in bacteria outside of clostridia.

Negative staining images of the native sample and 2D classification should be presented.

We have now added a figure to the Expanded View section (Fig. EV5B and C) which shows the previously published negative stain EM samples of TcdA at neutral and low pH (Pruitt et al., 2010) in comparison to high-resolution structures of TcdA and TcdB. In addition, to illustrate that our native TcdA sample at neutral pH is comparable to the corresponding negative staining EM TcdA sample, we have added several 2D classes from our cryo-EM data to Fig. EV1B in the Expanded View section.

In line 133 "from residue 2 to 2383 of the complete 2710 residues constituting the full-length TcdA". Is there only one class in final model?

The last heterogenous refinement run resulted in only one good 3D class of TcdA with the two other 3D classes being composed of junk particles and/or more conformationally flexible forms of TcdA. The good 3D class was used for a final homogeneous refinement resulting in the final TcdA map. The 2D classes calculated at an early stage in the processing workflow, where representative classes are shown in figure EV1B, were consistent with one major structural conformation of TcdA with pronounced flexibility observed from approximately residue 2283 of the CROPs domain. The region extending from residue 2283 of the TcdA CROPs domain was visible in the 2D classes but averaged out in the final 3D map.

Referee #3:

In this manuscript, Aminzadeh and colleagues present a high-resolution cryoEM-structure of TcdA, a large AB-type toxin from Clostridioides difficile. TcdA is the "little brother" of the closely related TcdB, but with the length of over 2700 amino acids, it still is an intimidatingly large protein (at least for a protein crystallographer such as this referee) that has imposed enormous challenges to the community of structural biologists before structures of the complete protein could be determined some years ago (my group and I have utterly failed ourselves some while back). With few notable exceptions for the TcdA/B toxins, where some crystal structures have been published recently, work with such large proteins has been revolutionized by the advent of new hardware for cryoEM structure determination, and the manuscript by Aminzadeh et al. sets an excellent example for this.

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We have added a multi-step mechanism figure to the manuscript (Fig. 1B).

- L82/86: I am personally not a fan of the word "solved" when it comes to talking about crystal structures - it is lab jargon referring to the step of initial phasing rather than the whole process of structure determination. I therefore prefer "determined", but I leave it up to the authors to reword the text or not.

We agree with the reviewer and have changed ”solved” to ”determined” throughout the manuscript. Thanks.

- L144: delete comma after pH

Done.

- Use of "CROPs" and "CROPs domain": it is not always clear if the text refers to the complete "tail" of the protein (in which case I think that "CROPs domain" suites better) or to single building blocks of it or even to peptides within these blocks. As a consequence, some sentences read as if there is a mix-up of singular and plural (e.g. in L253). The authors may want to think about the wording in these places carefully throughout the manuscript.

The use of ”CROPs domain” is now kept consistent throughout the revised manuscript, and refers to the complete tail of the protein. The word ”CROPs” has been used in a few places, which refers to only a part of the complete CROPs domain and is now specified in each sentence. Hope this makes it less confusing.

- L305/306: the authors state that their TcdA structure is in line with an older low-resolution structure of Pruitt et al. from 2010 - I wonder if this could be shown in a (supporting) figure.

We have added a new supporting figure (Fig. EV5B and C) to the Expanded View section illustrating this point.

- L333/334: the authors state that the contacts found in their new TcdA structure "prevent premature pore formation until reaching the endosomal compartment". I wonder what they envision here: I guess that if one simply lowers the pH of a solution containing these toxins, the proteins will simply precipitate due to aggregate formation after exposing hydrophobic regions without having a membrane into which these regions could be inserted. Hence, there would be no "premature pore formation". Or do they envision insertion into the plasma membrane of the cell? I guess none of us knows what these pores look like as yet - is one copy of the protein enough to establish translocation, or do several proteins have to come together? Is the translocated cargo unfolded or not?

We have changed the phrase ”premature pore formation” to ”premature conformational changes”, and also added a sentence clarifying the point we are making (line 527-530). Furthermore, we have included some of the speculations from this comment to the discussion (line 601-602).

- The manuscript evolves around comparisons with previously published structures. Without knowing details of this work, but maybe the authors can obtain even more insight by also discussing a recently published paper by Peng Chen et al. (doi: 10.1038/s41467-021-23878-3.), which shows the structure of a fragment of TcdB in complex with a receptor.

We are now discussing insights from this paper in the revised manuscript (line 628-649).

Dear Dr. Jørgensen,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of the manuscript. However, all three referees have suggestion to improve the manuscript I ask you to address in a final revised version of the manuscript. Please also provide a brief response addressing these points of the referees. As all three referees indicate grammar issues, please have your final manuscript carefully proofread by a native speaker.

Moreover, I have these editorial requests:

- We plan to publish your manuscript in the 'Report, format (as you also indicate in the submission system). For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Please add the full links for the deposited datasets to the DAS (data avalibility section) and make sure the data are public upon publication of the paper.

- Could some more information be added to the legend of Figure EV1A. What are the two images shown? Please consider that this needs to be understood also by non-specialist readers. Moreover, could scale bars be added to the images shown in EV1B?

- There is a callout to a Supplementary Table S1 in the text. Please update the nomenclature. Is this Table EV1?

- Please remove EV Table 1 from the manuscript text file. Please upload this separately upon re-submission as 'Expanded View' item.

- Finally, please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for co-corresponding author Thomas Boesen. We will not proceed with publication if this is not done. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines: http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short 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.

Best,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This revision of the initial paper has addressed some of the initial issues, but not the main request of a low pH Cryo-EM model for TcdA made by two reviewers. Since the authors responded that it was not possible to general a low pH Cryo-EM model for TcdA due to technical difficulties, some mention needs to be made regarding these difficulties in the manuscript. Otherwise, it is the first glaring question that comes to mind given the history in the field. In addition, the paper implies though does not explicitly state that TcdB is a better choice for comparison. If this is not the intent, then the inclusion of a short sentence regarding technical difficulties is not unwarranted even if we all hate admitting a 'negative result'.

Overall, the paper more clearly lays out the conclusions though this reviewer wishes there was a clearer way to delineate between the crystal structure, TcdAx, and the cryo-EM structure, TcdA, than the mere addition of an x.

Very minor points which require revision:

Naming the Melnyk group (L378, L390, L407) and the Lacy group (L384) seems an unnecessary change from simply referencing

the papers (as seen in version 1) especially as the PIs of other cited papers are not referred to.

L405, argue is a lonely verb in need of a subject like 'this' and then argue should be conjugated properly for the subject such as 'this argues'.

L409-L410, "The TcdA structure also explains the inability of TcdA to bind to the frizzled-protein and Chondroitin Sulfate Proteoglycan 4 (CSPG4) receptors shown to bind to TcdB" is an incomplete sentence. It needs something between "and Chondroitin" or some justification for "Chondroitin Sulfate Proteoglycan 4 (CSPG4) receptors shown to bind to TcdB". I don't know what the authors are trying to say here. Perhaps, "and this is why Chondroitin Sulfate Proteoglycan 4 (CSPG4) receptors are shown to bind to TcdB"?

L410, Why is Frizzled protein hyphenated in the previous sentence but not here? I would pick one or the other, but not both.

Referee #2:

The revised manuscript has addressed reviewers' comments adequately.

A few minor suggestions:
1. On page 18, line 468: "based on an initial blop picking" should be "based on an initial blob picking".
2. On page 32, Table EV1, Refinement, "FSC threshold" value is missing?
3. Some of the discussion section can be moved and integrated into the "Result" section, for instance, lines 327-333.

Referee #3:

Aminzadeh and colleagues revised their manuscript "High-resolution structure of native toxin A from Clostridioides difficile", which had first been submitted in summer this year. While I already enjoyed reading the original draft, I did so even more with this new version since the authors have taken most of the criticism of all three referees seriously, with the exception of collecting new data of a TcdA investigated at low pH. While the request of my referee colleagues to obtain such a structure with the improved cryoEM equipment that has become available since such a structure was reported at low resolution several years back is certainly reasonable, the authors' claim that they have tried but could not optimize the respective samples in the time allowed for revising the paper is understandable. It seems possible that simply acidifying the toxin is not sufficient, one could envision that membrane components/detergents are required for such experiments.

The manuscript is well written and illustrated, the only point that felt weird is the authors' use or not-use of commas in places, e.g. in lines 29/39, 47, 48, 52, 53, 66, 176, 286, 376 to name a few that I have marked. There are also a few mix-ups of singulars and plurals, e.g. in lines 21, 41, 117, 387. However, these are just minor points that may also get corrected by the copy editor of the manuscript.

November 9, 2021

Point-by-point response

Please note that line numbers mentioned below refer to the line numbers in the attached word document with track changes as these are different from the numbers in the converted PDF file.

Editor´s comment: Authors' response in blue

- Please add the full links for the deposited datasets to the DAS (data avalibility section) and make sure the data are public upon publication of the paper.

Line 849-850: A link covering both the map and the coordinates are now added to the Data Availability Section.
The deposition is currently on hold will be released as soon as the manuscript is officially accepted.

- Could some more information be added to the legend of Figure EV1A. What are the two images shown? Please consider that this needs to be understood also by non-specialist readers. Moreover, could scale bars be added to the images shown in EV1B?

Line 1127-1135: More information has been added to the Figure EV1A legend with focus on the two images and that it should be clear to a non-specialist.

- There is a callout to a Supplementary Table S1 in the text. Please update the nomenclature. Is this Table EV1?

You are correct. Supplementary Table S1 should be Table EV1. This has been corrected on line 847.

- Please remove EV Table 1 from the manuscript text file. Please upload this separately upon re-submission as 'Expanded View' item.

This is done.

- Finally, please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for co-corresponding author Thomas Boesen. We will not proceed with publication if this is not done. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines: http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

Thomas Boesen has now supplied his ORCID ID

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).

Summary

We report a high-resolution cryo-EM structure of the TcdA toxin from *Clostridioides difficile* at neutral pH. The structure provides insights into the pH-induced inter-domain dynamics and mechanism of preventing premature unfolding of the pore-forming region.

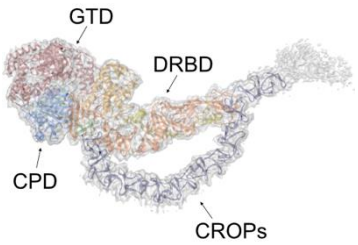
- two to four short bullet points highlighting the key findings of your study.

Bullet Points

- The article describes the first high-resolution cryo-EM structure of full-length native TcdA from *Clostridioides difficile.*
- The structure reveals detailed information about the interaction between the CROPs domain and the tip of the delivery and receptor-binding domain and uncovers the pH-induced dynamic movement of the CROPs domain.
- Furthermore, the structure provides insights into the mechanism of preventing premature unfolding of the pore-forming region at neutral pH.

- 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.

The shown schematic summary figure has been uploaded in the submission process in tiff format and with the requested dimensions.



Finally, we have moved Figure EV4 to the main text which is now Figure 4. In addition, we have split figure EV4 up into EV2 and EV5.

Referee #1:

This revision of the initial paper has addressed some of the initial issues, but not the main request of a low pH Cryo-EM model for TcdA made by two reviewers. Since the authors responded that it was not possible to general a low pH Cryo-EM model for TcdA due to technical difficulties, some mention needs to be made regarding these difficulties in the manuscript. Otherwise, it is the first glaring question that comes to mind given the history in the field. In addition, the paper implies though does not explicitly state that TcdB is a better choice for comparison. If this is not the intent, then the inclusion of a short sentence regarding technical difficulties is not unwarranted even if we all hate admitting a 'negative result'.

We now describe our unsuccessful attempt at obtaining a high-resolution structure at low pH in the Materials and Methods section (Line 794-795 and 829-830) as well as commenting on this in the Results (Line 164-168) and argue that using TcdB instead can be justified (Line 184-186 and 196-198).

Overall, the paper more clearly lays out the conclusions though this reviewer wishes there was a clearer way to delineate between the crystal structure, TcdAx, and the cryo-EM structure, TcdA, than the mere addition of an x.

We have replaced the TcdAx crystal structure reference with $TcdA_{1832,}$ which is also the reference used in the publication of the crystal structure (Chumbler et al., 2016).

Very minor points which require revision:

Naming the Melnyk group (L378, L390, L407) and the Lacy group (L384) seems an unnecessary change from simply referencing the papers (as seen in version 1) especially as the PIs of other cited papers are not referred to.

We have removed all references to specific groups.

L405, argue is a lonely verb in need of a subject like 'this' and then argue should be conjugated properly for the subject such as 'this argues'.

We have rephrased the sentence to the following;

Line 641-644: “Therefore, since the hinge SR has a similar sequence motif, is structurally similar to the other CROPs SRs and moves in conjunction with the CROPs domain as a unit, **we propose to** expand the CROPs domain to also include this hinge SR starting from residue Leu1811.”

L409-L410, "The TcdA structure also explains the inability of TcdA to bind to the frizzled-protein and Chondroitin Sulfate Proteoglycan 4 (CSPG4) receptors shown to bind to TcdB" is an incomplete sentence.
It needs something between "and Chondroitin" or some justification for "Chondroitin Sulfate Proteoglycan 4 (CSPG4) receptors shown to bind to TcdB". I don't know what the authors are trying to say here. Perhaps, "and this is why Chondroitin Sulfate Proteoglycan 4 (CSPG4) receptors are shown to bind to TcdB"?

We have rewritten the sentence to the following;
Line 647-649: “The TcdA structure also explains why TcdA is unable to bind to the frizzled protein and CSPG4 receptors, which are previously described structurally in crystal structure complexes of TcdB and receptor (Chen et al, 2018, 2021).”

L410, Why is Frizzled protein hyphenated in the previous sentence but not here? I would pick one or the other, but not both.

We have removed the hyphenation in the first sentence.

Referee #2:

1. On page 18, line 468: "based on an initial blop picking" should be "based on an initial blob picking".

Line 846: This has been corrected.

2. On page 32, Table EV1, Refinement, "FSC threshold" value is missing?

The missing FSC threshold value in Table EV1 under Refinement is now added

3. Some of the discussion section can be moved and integrated into the "Result" section, for instance, lines 327-333.

These sections have been either deleted, moved to the results section or rephrased to fit the discussion section. (These modifications are indicated in the track changes of the manuscript)

Referee #3:

The only point that felt weird is the authors' use or not-use of commas in places, e.g. in lines 29/39, 47, 48, 52, 53, 66, 176, 286, 376 to name a few that I have marked. There are also a few mix-ups of singulars and plurals, e.g. in lines 21, 41, 117, 387. However, these are just minor points that may also get corrected by the copy editor of the manuscript.

We have corrected the bad grammar pointed out by the Referee and in addition the manuscript was carefully proofread by a native speaking person.

Dr. René Jørgensen
Statens Serum Institut
Microbiology and Infection Control
Artillerivej 5
Copenhagen 2300
Denmark

Dear Dr. Jørgensen,

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.

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Yours sincerely,

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Editor
EMBO Reports

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Corresponding Author Name: René Jørgensen
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53597V1

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 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 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?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA

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Is there an estimate of variation within each group of data?	NA
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).	NA
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	Protein Data Bank entries for the Cryo-EM map and the TcdA model (PDB ID 7POG and EMD-13574) are written in the Data Availability section
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