Plasma membrane protrusions mediate host cell-cell fusion induced by *Burkholderia thailandensis*

Nora Kostow and Matthew Welch

*Corresponding author(s): Matthew Welch, University of California, Berkeley*

---

**Review Timeline:**

| Event                    | Date       |
|--------------------------|------------|
| Submission Date          | 2022-02-25 |
| Editorial Decision       | 2022-03-21 |
| Revision Received        | 2022-05-06 |
| Accepted                 | 2022-05-07 |

*Editor-in-Chief: Thomas Pollard*

**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.)
Dear Authors:

Our two expert reviewers feel that your work is valuable and a good candidate for publication in MBoC. Both have some thoughtful advice about how to improve the presentation and strengthen your case regarding the mechanism.

I suggest that you consider enlarging many of your postage stamp-sized fluorescence micrographs so that readers can see the details you describe. For example, you might make Fig 1 a two-column figure. In Fig 2 the graphs are more than adequate in size while the micrographs are too small. At the very least use the full single column width rather than leaving blank space at the upper right. Fig 4 has the same problem with oversized graphs and undersized micrographs.

We look forward to receiving a revised version of your paper that addresses constructively each of the points raised in review.

Sincerely,

Thomas Pollard
Monitoring Editor
Molecular Biology of the Cell

------------------------------------------------------------------------

Dear Dr. Welch,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor’s decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor’s and reviewers’ comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
Reviewer #1 (Remarks to the Author):

In this manuscript, Kostow and Welch investigated the molecular machinery mediating host cell to cell fusion induced by Burkholderia thailandensis. Using live imaging, the authors showed that bacteria used actin-based motility to generate plasma membrane protrusions that extended into neighboring cells. About 60% of cell membrane fusion events occurred at the tip of the protrusions, while 40% of the fusion event happened elsewhere on the protrusions. There was no correlation between the length of the protrusions and how fast fusion occurred or where fusion occurred, but cell-cell fusion did occur faster when it happened at the tip of protrusions than elsewhere. The authors further studied at which step VgrG5, which is known to be necessary for bacteria-induced host cell-cell fusion, acts to promote cell-cell fusion and found that it is not required for protrusion formation but for the membrane fusion step. Moreover, VgrG5 acted as a secretory protein and must be secreted within a protrusion to induce cell-cell fusion. In addition to VgrG5, the authors also found that TagD5, the PAAR protein associated and co-secreted with VgrG5, is also required for cell-cell fusion and acts at the membrane fusion step.

Overall, this is a nice study demonstrating an essential role for invasive membrane protrusions and T6SS-5 proteins in B. thailandensis-induced cell-cell fusion. Experiments are well designed and conducted, and the conclusions are largely supported by the data presented. I only have a few comments.

1. In Video S2, it is unclear if fusion between the two cells was indeed induced by the bacterium at the top, because the cell membranes between the two cells appeared to have a gap even before that bacterium made the protrusion. Given the time lag between fusion pore formation at the tip of the protrusion and GFP diffusion shown in Video S1, is it possible that another bacterium already induced fusion pore formation between the two cells before the start of the video? This is an important point, because the authors concluded that 40% of the fusion pores form at a distance from the bacteria. It would be helpful if the authors could provide two videos of each scenario (fusion pore at tip or elsewhere).

2. If fusion could happen at the tip or elsewhere in the protrusion, why is there a time difference for cytoplasmic mixing between the two modes - when fusion happens at the tip, there is a time lag for cytoplasmic mixing, whereas when fusion happens at elsewhere, there does not seem to be a lag.

3. It is unclear how the secreted proteins, VgrG5 and TagD5, may induce cell membrane fusion. Where are they localized within the protrusions, and is there any correlation between the localization of VgrG5/TagD5 and the position of the fusion pore?

4. In Figure 3, the authors used the VgrG5 wild-type bacteria to perform rescue experiment and found that wild-type bacteria inside the host cell failed to rescue the fusion defect. Based on this result, the authors concluded that VgrG5 must be secreted within the protrusion to induce cell-cell fusion. However, if VgrG5 can only be secreted by bacteria in protrusions, it is not surprising that wild-type bacteria in the cytosol failed to rescue the defect.

5. Why is protrusion engulfment in the TagD5 mutant faster than in the VgrG5 mutant? Is it because in the TagD5 mutant, VgrG5 can be secreted and contribute to the engulfment process?

6. In the Introduction, more up-to-date reviews should be cited on the function of the cytoskeleton and adhesion molecules in cell-cell fusion.

Reviewer #2 (Remarks to the Author):

General comment

While the ultimate outcome of "cell-cell fusion" is unquestionable, this reviewer is challenging the interpretation put forth by the authors with respect to "membrane fusion". The authors did not use techniques that would unambiguously show membrane fusion in protrusions, such as electron microscopy on serial sections. Given the resolution of the provided images, there are alternative interpretations, including massive pore formation without membrane repair. It is therefore suggested that the authors tone down the wording in the Results section, staying away from over-interpreting the data and carefully reporting what they observed, including protrusion formation, bacteria exiting the formed protrusions, and correlation with subsequent cell-cell fusion. Then, it is appropriate to speculate in the Discussion section that one mechanistic interpretation of the results could be VgrG5-mediated membrane fusion in protrusions.
Specific Comments

Figure 1 leaves the reader under the impression that any event of protrusion formation leads to cell-cell fusion. Could the authors provide quantitative analyses of the proportion of the formed protrusions that leads to cell-cell fusion, and the proportion of cell-cell fusion events that correlates with protrusion formation?

The events reported in the text and corresponding to the host cell GFP channel are difficult to discern in the images presented in Figure 1A. Perhaps showing the GFP channel only would help? Of note, GFP diffusion is much more convincing in the corresponding movies.

With respect to membrane fusion occurring in protrusions as inferred from cytosolic GFP leakage, it is important to show the host cell GFP channel only corresponding to the "exit protrusion" image in Figure 1A and its graphical interpretation in Figure 1B. Showing that GFP diffuses from the tip of the formed protrusion would convincingly suggest that GFP "leakage" occurs in protrusion.

In movie S1, it appears that, as the bacterium pierces through the protrusion tip, the membrane remnant retracts towards the donor cell, while the membrane marker concomitantly vanishes from cell-cell contact, as the protrusion is still clearly visible. This may suggest a global membrane disruption event at cell-cell contact as opposed to the proposed local fusion event in protrusion. Please comment.

Assuming the authors’ goal in Figure 3 was to determine if delivery of Vgrg5 to the plasma membrane by WT bacteria could rescue in trans the vgrg5 mutant in its own protrusions, then the experimental design is problematic. At best, the conducted experiment shows that, in the cells imaged by the authors, Bt BFP WT did not deliver VgrG5 to the plasma membrane upon spread, otherwise the authors would have observed cell-cell fusion. So, by design, these complementation experiments could not unambiguously demonstrate that VgrG5 is locally delivered/required in protrusions. The authors should consider a more direct approach using tagged-versions of VgrG5 to visualize the protein in protrusion membrane. This reviewer understands this approach may be challenging, but functional, HA-tagged versions exist, and allow for detection of VgrG5, at least in vitro. So, it is worth trying immuno-staining on fixed samples using the tissue culture system used by the authors.

In the Discussion section, the statement "protrusion formation through actin-based motility is sufficient to mediate cell-to-cell spread" is quite a stretch given the abundant literature, including seminal papers from the Welch lab, demonstrating that, beyond actin-based motility, bacterial spread from cell to cell is supported by bacterial and host cell factors.
Response to Monitoring Editor

We thank the Monitoring Editor for their comments and have carried out their suggested changes to the sizing of figures and graphs.

Response to Reviewers

We thank the reviewers for taking the time to thoroughly read and review our manuscript and for making very thoughtful and constructive comments. The comments helped us incorporate key improvements into this revised manuscript.

Reviewer #1 (Remarks to the Author):

“In this manuscript, Kostow and Welch investigated the molecular machinery mediating host cell to cell fusion induced by Burkholderia thailandensis. Using live imaging, the authors showed that bacteria used actin-based motility to generate plasma membrane protrusions that extended into neighboring cells. About 60% of cell membrane fusion events occurred at the tip of the protrusions, while 40% of the fusion event happened elsewhere on the protrusions. There was no correlation between the length of the protrusions and how fast fusion occurred or where fusion occurred, but cell-cell fusion did occur faster when it happened at the tip of protrusions than elsewhere. The authors further studied at which step VgrG5, which is known to be necessary for bacteria-induced host cell-cell fusion, acts to promote cell-cell fusion and found that it is not required for protrusion formation but for the membrane fusion step. Moreover, VgrG5 acted as a secretory protein and must be secreted within a protrusion to induce cell-cell fusion. In addition to VgrG5, the authors also found that TagD5, the PAAR protein associated and co-secreted with VgrG5, is also required for cell-cell fusion and acts at the membrane fusion step."

Response: We thank the reviewer for the thoughtful and thorough summary of the work.

“Overall, this is a nice study demonstrating an essential role for invasive membrane protrusions and T6SS-5 proteins in B. thailandensis-induced cell-cell fusion. Experiments are well designed and conducted, and the conclusions are largely supported by the data presented. I only have a few comments.”

Response: We thank the reviewer for their positive comments.

“1. In Video S2, it is unclear if fusion between the two cells was indeed induced by the bacterium at the top, because the cell membranes between the two cells appeared to have a gap even before that bacterium made the protrusion. Given the time lag between fusion pore formation at the tip of the protrusion and GFP diffusion shown in Video S1, is it possible that another bacterium already induced fusion pore formation between the two cells before the start of the video? This is an important point, because the authors concluded that 40% of the fusion pores form at a distance from the bacteria. It would be helpful if the authors could provide two videos of each scenario (fusion pore at tip or elsewhere).”

Response: We thank the reviewer for the suggestion. To address this comment, we have taken the reviewer’s suggestion and included two examples of each type of pathway. To create more room to show both examples and to expand the size of the images as suggested by the
Monitoring Editor, we have split the prior Figure 1 into revised Figures 1, 2 and 3. In Video S2 we were lucky to capture the cell for 27 min 15 s before the bacterium approached the plasma membrane. No other membrane protrusions formed in that cell prior to the one shown in the video, so we are confident that the fusion originated from that protrusion and that any “gap” in the plasma membrane that might appear is likely due to the variability in appearance of the host plasma membrane marker, as there was no discernable leakage of GFP signal from one cell into the other. For quantification, we only analyzed videos in which we could confidently assess the sequence of events leading to cell-cell fusion, including: which membrane protrusion caused the fusion, time of protrusion formation, time of GFP diffusion, and time of bacterial exit from the protrusion. This is now described more explicitly in the text.

“2. If fusion could happen at the tip or elsewhere in the protrusion, why is there a time difference for cytoplasmic mixing between the two modes - when fusion happens at the tip, there is a time lag for cytoplasmic mixing, whereas when fusion happens at elsewhere, there does not seem to be a lag.”

**Response:** We found that fusion that occurs at the protrusion tip results in slightly faster cytoplasmic mixing than for fusion elsewhere in the protrusion (see new Figure 3E). We agree with the reviewer that this difference is intriguing, but it is not clear at this time why that is the case.

“3. It is unclear how the secreted proteins, VgrG5 and TagD5, may induce cell membrane fusion. Where are they localized within the protrusions, and is there any correlation between the localization of VgrG5/TagD5 and the position of the fusion pore?”

**Response:** We agree with the reviewer, as well as Reviewer #2, that VgrG5 and TagD5 localization data would help clarify their mechanism of action. However, efforts to visualize FLAG tagged VgrG5 using anti-FLAG antibody or native VgrG5 using anti-VgrG5 antibody have not yielded results. We hypothesize that VgrG5, if it is indeed secreted, is present in too low abundance to be visualized by these methods. Therefore, localization of VgrG5 will not be feasible to include in this work.

“4. In Figure 3, the authors used the VgrG5 wild-type bacteria to perform rescue experiment and found that wild-type bacteria inside the host cell failed to rescue the fusion defect. Based on this result, the authors concluded that VgrG5 must be secreted within the protrusion to induce cell-cell fusion. However, if VgrG5 can only be secreted by bacteria in protrusions, it is not surprising that wild-type bacteria in the cytosol failed to rescue the defect.”

**Response:** We appreciate this comment, as well as a similar comment from Reviewer #2 about our interpretation of this result. In response to this comment, we restrict our interpretation of this result to the Discussion so as not to overreach in our conclusions in the Results section. Because we cannot determine when/where/whether VgrG5 is secreted, we cannot make any statements about VgrG5 secretion. Based on this experiment, we now claim that VgrG5 must be “expressed” rather than “secreted” by bacteria in protrusions in order for that bacterium to induce cell-cell fusion. Our results are consistent with VgrG5 being secreted within protrusions but we will restrict this interpretation to the Discussion.

“5. Why is protrusion engulfment in the TagD5 mutant faster than in the VgrG5 mutant? Is it because in the TagD5 mutant, VgrG5 can be secreted and contribute to the engulfment
Response: The timing of protrusion engulfment for the TagD5 mutant was not statistically significantly different from engulfment for the VgrG5 mutant. This data is shown in Supplemental Figure 4C. We have added a sentence to the results section to clarify this result.

“6. In the Introduction, more up-to-date reviews should be cited on the function of the cytoskeleton and adhesion molecules in cell-cell fusion.”

Response: We appreciate this comment and have selected 3 additional reviews to cite that were published in 2017, 2019, and 2020.

Reviewer #2 (Remarks to the Author):

“General comment
While the ultimate outcome of "cell-cell fusion" is unquestionable, this reviewer is challenging the interpretation put forth by the authors with respect to "membrane fusion". The authors did not use techniques that would unambiguously show membrane fusion in protrusions, such as electron microscopy on serial sections. Given the resolution of the provided images, there are alternative interpretations, including massive pore formation without membrane repair. It is therefore suggested that the authors tone down the wording in the Results section, staying away from over-interpreting the data and carefully reporting what they observed, including protrusion formation, bacteria exiting the formed protrusions, and correlation with subsequent cell-cell fusion. Then, it is appropriate to speculate in the Discussion section that one mechanistic interpretation of the results could be VgrG5-mediated membrane fusion in protrusions.”

Response: We appreciate the reviewer’s careful interpretation of our data. We have toned down our wording in the Results section, removing mention of “fusion pore formation,” which we cannot directly visualize, and instead using the general term “membrane fusion” because it describes the end result of the pathway of cell-cell fusion, even if we do not yet know the intermediate steps. As the reviewer suggests, in the Discussion section we then speculate on the possibility of VgrG5-mediated membrane fusion.

“Specific Comments
Figure 1 leaves the reader under the impression that any event of protrusion formation leads to cell-cell fusion. Could the authors provide quantitative analyses of the proportion of the formed protrusions that leads to cell-cell fusion, and the proportion of cell-cell fusion events that correlates with protrusion formation?”

Response: We appreciate the reviewer’s comment. With regards to the first part of the comment, it is clear that sometimes protrusion formation does not lead to cell-cell fusion, as we observed protrusions that were engulfed (see Supplemental Figure 4) or otherwise did not lead to cell-cell fusion. We speculate that this could be due to a deficiency of T6SS-5 function by those bacteria. With regards to quantification of the proportion of formed protrusions that unambiguously lead to cell-cell fusion, we are working with a dataset of 20 examples where it is clear which protrusion led to the cell-cell fusion, and in that case it is 100%. To address the correlation of cell-cell fusion events that correlate with protrusions, in the revised manuscript we now include more information about the number of cell-cell fusion events observed (144), the
number correlated with membrane protrusions (141), and the number with no obvious membrane protrusions (3). We also include a description of how the dataset of 20 was selected from the 144 cell-cell fusion events observed. Hopefully these changes give the reviewer and reader a better sense of how the selected examples relate to the overall data collected.

“The events reported in the text and corresponding to the host cell GFP channel are difficult to discern in the images presented in Figure 1A. Perhaps showing the GFP channel only would help? Of note, GFP diffusion is much more convincing in the corresponding movies.”

Response: We thank the reviewer for this suggestion. In the revised manuscript we include insets that show the GFP channel alone (in greyscale) and with increased brightness so that the reader can more easily see the GFP diffusion.

“With respect to membrane fusion occurring in protrusions as inferred from cytosolic GFP leakage, it is important to show the host cell GFP channel only corresponding to the "exit protrusion" image in Figure 1A and its graphical interpretation in Figure 1B. Showing that GFP diffuses from the tip of the formed protrusion would convincingly suggest that GFP "leakage" occurs in protrusion.”

Response: We appreciate the reviewer’s comment and agree that the cartoons did not mimic what is being observed in the videos. To address this comment, we have adjusted the cartoons to more closely match the observable GFP diffusion and remove our interpretation that the GFP diffusion originates from a particular point. With regards to the last part of this comment, the origin of GFP diffusion cannot be determined by this method as the GFP diffuses quickly into the whole cell so we do not see it emanating from a particular location.

“In movie S1, it appears that, as the bacterium pierces through the protrusion tip, the membrane remnant retracts towards the donor cell, while the membrane marker concomitantly vanishes from cell-cell contact, as the protrusion is still clearly visible. This may suggest a global membrane disruption event at cell-cell contact as opposed to the proposed local fusion event in protrusion. Please comment.”

Response: We appreciate the reviewer’s comment and agree that the membrane label is difficult to interpret in this example. However, we feel confident claiming that fusion occurred locally at the tip of the protrusion based on the bacterium exiting the protrusion at this location. Nevertheless, this does not rule out the possibility that cell-cell fusion also occurred elsewhere within the protrusion. To address this comment and a comment from Reviewer #1, we now included two examples for each cell-cell fusion pathway instead of one (Figures 1 and 2). Hopefully these examples more convincingly illustrate the point that cell-cell fusion occurs within protrusions, emanates outward from protrusions to the rest of the cell periphery, and can happen at the protrusion tip or elsewhere within the membrane protrusion. While we suspect a model in which cell-cell fusion is mediated by a fusogen and occurs through a hemifusion pathway leading to a fusion pore, as all other known cell-cell fusion pathways do, our results cannot determine the molecular-level mechanism of cell-cell fusion (including a "global membrane disruption" hypothesis put forth by this reviewer). We have added an additional sentence to the discussion to address this: “Although our results are insufficient to determine the molecular-level mechanism of membrane fusion during B. thailandensis induced cell-cell fusion, they are consistent with membrane fusion involving a canonical fusogen-mediated hemifusion pathway (Hernández & Podbilewicz, 2017).”
“Assuming the authors’ goal in Figure 3 was to determine if delivery of VgrG5 to the plasma membrane by WT bacteria could rescue in trans the vgrg5 mutant in its own protrusions, then the experimental design is problematic. At best, the conducted experiment shows that, in the cells imaged by the authors, Bt BFP WT did not deliver VgrG5 to the plasma membrane upon spread, otherwise the authors would have observed cell-cell fusion. So, by design, these complementation experiments could not unambiguously demonstrate that VgrG5 is locally delivered/required in protrusions. The authors should consider a more direct approach using tagged-versions of VgrG5 to visualize the protein in protrusion membrane. This reviewer understands this approach may be challenging, but functional, HA-tagged versions exist, and allow for detection of VgrG5, at least in vitro. So, it is worth trying immuno-staining on fixed samples using the tissue culture system used by the authors.”

Response: We appreciate this reviewer’s (and Reviewer #1’s) attention to interpreting this experiment. Because we cannot determine when/where/whether VgrG5 is secreted, we do not claim that VgrG5 is delivered to the plasma membrane and this experiment was not designed to test this hypothesis. We have changed the text of the Results section to conclude that VgrG5 must be “expressed by bacteria in protrusions” rather than “secreted in protrusions” in order to induce fusion. In the Discussion section we then propose that VgrG5 is likely to be secreted in protrusions, localize to protrusions, and function within protrusions. We agree with the reviewer that VgrG5 and TagD5 localization data would help clarify their mechanism of action. To our knowledge, there are no published studies describing functional tagged versions of VgrG5 (the HA tagged VgrG5 presented in Toesca et al. 2014 does not support cell-cell fusion and there was no data on cell-cell fusion presented in Schwarz et al. 2014 about the VgrG5-VSV-G tag used). We identified an internal region of VgrG5 that could be functionally tagged with a FLAG tag, but unfortunately, our efforts to visualize VgrG5 using an anti-FLAG antibody or anti-VgrG5 antibody have not yielded localization results. We hypothesize that VgrG5, if it is indeed secreted, is in too low abundance to be visualized by these methods. Therefore, localization of VgrG5 will not be feasible to include in this work.

“In the Discussion section, the statement "protrusion formation through actin-based motility is sufficient to mediate cell-to-cell spread" is quite a stretch given the abundant literature, including seminal papers from the Welch lab, demonstrating that, beyond actin-based motility, bacterial spread from cell to cell is supported by bacterial and host cell factors.”

Response: We appreciate the reviewer’s comment. To address this, we have reworded that sentence to read: “Our observations are also consistent with prior observations that protrusion formation through actin-based motility drives bacterial engulfment into the recipient cell (Monack & Theriot, 2001)".
Dear Matt:

Many thanks for the clear, constructive responses to the reviewers. The micrographs look far better at higher magnification. I am satisfied with your revisions and pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Tom

Thomas Pollard
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Welch:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org