nature portfolio

Peer Review File

Super-resolving microscopy reveals the localizations and movement dynamics of stressosome proteins in Listeria monocytogenes

Response to reviewers

Reviewer #1 (Remarks to the Author):

The authors measured the diffusion of serval proteins related to the stressosomes in Listeria monocytogenes. Although the techniques employed are modern and experiments are carefully done, the results do not form a cogent story that provides new insights into the structure or function of stressosomes. Thus, this work may not be suitable for Nature Communications.

Specifically, the authors used a fluorescent protein, mEos3.2, to label the different proteins and measured diffusion. How are their diffusion measurements, which seem to be based on the discussion of individual proteins, relate to the "RsbR-RsbS 60-protomers truncated icosahedron" structure of the stressosome? Their PALM images of mEos3.2::rsbR1 did not appear to be integrated into clusters that can be identified as stressosomes.

>>> Reply: We thank the reviewer for the appreciation of our work, in particular the technical aspects of the advanced microscopy and data analysis. We disagree with the reviewer that we do not present a compelling story. We acknowledge that some results are surprising and differ from the current thinking, e.g. the membrane-bound state of RsbR1 is not required for sensing, and RsbL forms clusters upon illumination. We feel that this emphasises the importance of our measurements, which are complementary to classical molecular biology and microbiology approaches used in the past.

We studied the dynamics of stressosome proteins in live cells, and we infer from the diffusion data whether the proteins form higher-order assemblies such as stressosomes or bind to the membrane. We see no other direct way of measuring the dynamics of stressosome complexes; one always has to tag one of the subunits. The RsbR-RsbS 60-protomer truncated icosahedron structure is composed of multiple subunits from the structural study[1] that can also be present in the cell as individual proteins or subcomplexes. It is the diversity of (sub-)structures that we have probed with advanced light microscopy techniques.

Indeed, it is surprising that, unlike mEos3.2-RsbL clustered upon illumination, mEos3.2-RsbR1 did not always integrate into one defined cluster size,

corresponding to the full stressosome complex, but that sub-complexes can be found as well (Supplementary Video 1 and Supplementary Video 2). We have modified the text to explain the approach and observations better.

Additional issues with the major conclusions:

1. A local heterogeneity of diffusion in the cytoplasm of *L. monocytogenes* with free mEos3.2 proteins in the middle of the cell diffusing faster than in the pole regions. This is not related to the stressosome topic of this work.

>>> Reply: We agree on the point of local heterogeneity, and we have no longer accentuated the differences in the mobility of mEos3.2 in the pole regions and middle of the cell as one of the main findings. However, the measurements on free mEos3.2 are essential. It is a benchmark for the fusion constructs and comparing the protein diffusions in the cytoplasm and at the membrane.

2. the small hydrophobic protein Prli42 is essential for membrane localization of RsbR1 but does not influence stress sensing.

The results are not very convincing. For example, the D values were 0.2±0.04 $\mu m^2/s$ for the wild type and 0.29±0.11 $\mu m^2/s$ for the removal of prli42. Also, the pH=5 results of 0.14±0.02 $\mu m^2/s$ and 0.15±0.03 $\mu m^2/s$ were essentially the same between the wildtype and prli42 removal. They also showed PALM images of RsbR1, but even in the examples given, the differences in membrane localization are not very clear between the wildtype and prli42 removal.

>>> Reply: We fully understand the concern of the reviewer. The differences in diffusion coefficients are minor, yet they are significant - based on the confidence level of the statistical test ($P<0.001$). Notably, the SMdM and SPT data are internally consistent (Fig. 3f and Fig. 4g). The analysis of the protein diffusion that can be present in multiple states is complicated[2], and that is why we used different microscopy techniques (SMdM, SPT, FRAP and PALM) to substantiate our findings. We have modified the manuscript (Lines 227 – 234 and 255 - 256) by emphasizing the key findings, the significance of the data, and the intricacies of obtaining diffusion coefficients of proteins that can be present in different aggregation states. Additionally, Supplementary Videos 1 and 2 are added to show the localization and clustering of RsbR1.

3. The cytoplasmic fraction of RsbR1 is present in supramolecular complexes, presumably with RsbS (and probably RsbT) to form the stressosome;

These seem not directly supported by their diffusion data.

>>> Reply: We postulate the states of RsbR1 on the basis of its much slower diffusion than expected for a free cytosolic protein with Mw = 57.8 kDa (mEos3.2-RsbR1). We emphasise that the mEos3.2-RsbR1 fusion is in-frame with RsbS and RsbT both on the chromosome and plasmid. Hence, RsbR1 is likely present in the form of protomers of RsbR1-RsbS (but not in a full stressosome complex), which explains the slow diffusion. We have changed and assigned this state as the Intermediate state (Int), while monomeric mEos3.2-RsbR1 is the free state. We rephrased the text to clarify our interpretation of the data (Lines 280 - 290, 304 - 309, 451 – 454, and 468 – 470).

4. The slowing of diffusion of the blue light receptor RsbL upon illumination suggests that the association of the protein with the stressosome is blue-light dependent, a process that is independent of Prli42.

They used 405 and 560 nm. Which of the two illuminations played the role? Neither is “blue light”. Also, they should compare with another target like RsbR1 as a negative control to show no similar slowing of diffusion after illumination.

>>> Reply: The name blue-light receptor is given for RsbL (Lmo0799) in *L. monocytogenes* (and its homolog YtvA in *B. subtilis*) as it possesses a light, oxygen, voltage (LOV) domain that is homologous to the photoactive, flavin mononucleotide (FMN)-binding LOV domains of phototropin found in higher plants[3]. The photochemistry of RsbL shows an absorption spectrum in the ground state (dark state) with maxima near 375, 450, and 475 nm[3,4]. We used the laser lines of our microscopes to excite RsbL, which indeed does not correspond to the absorption maxima but suffice for photoconversion between the different states. We thus conclude that the clustering of RsbL, elicited by 405 nm illumination, is physiologically relevant.

As for the controls - we show in the manuscript (Lines 317 - 319 and 497 - 517) that the clustering upon laser illumination does not occur with free mEos3.2, mEos3.2-Prli42, and mEos3.2-RsbR1 (Supplementary Videos 1, 2, versus 3). Hence, the clustering of mEos3.2-RsbL is a genuine property of the light sensor RsbL.

Reviewer #2 (Remarks to the Author):

The authors of “The dynamics and function of stressosome proteins in Listeria monocytogenes” present thorough subcellular analysis of the location and diffusion of stressosome protein RsbR1 using a fluorescently tagged version. The techniques used allow for detailed and ensitive measurements that have not been shown before for the stressosome. Given the ubiquity of the stressosome in bacterial species and its “stress sensing hub” role, questions about the cellular and subcellular behavior of proteins in this complex are important ones to address. Overall the data of the mEose3.2::RsbR1 construct provide interesting observations about the subcellular localization and dynamics of RsbR1 (Fig3e, Fig3f, Fig4b, Fig4c) and the role that the membrane bound protein Prli42 plays in the diffusion of RsbR1. The results are consistent with those found previously in that RsbR1 interacts with Prli42 and the membrane association of RsbR1 is dependent onPrli42. Unfortunately, this reviewer found the data on RsbL less convincing and has suggestions on how to address it. Overall, the RsbR1 dynamic characterization will be useful for further testing a variety of SigB inducing conditions to understand stressosome dynamics.

>>> Reply: We thank the reviewer for his/her favourable evaluation of the paper and valid suggestions.

Recommendations.

1. Move the construct strain characterization text to the beginning of the results section. Lines 402-423. It is important to establish early on that the mEos3.2 fusions are functional so that the localization data are interpretable. The authors show that the chromosomal integrated RsbR1 construct phenocopies the WT in Supplementary figure 12. But the data on the RsbL construct are less favourable showing the construct is non-functional in its light sensing function, Supplementary Figure 13. Given that the characterization of the RsbL fusion is done using light irradiation, and cells containing the construct did not complement a null in a light sensing assay, the data on RsbL localization and clustering are less than convincing. This reviewer would recommend, removing the RsbL data all together or at the very least noting that the data are inconclusive and deemphasizing them. See #2 for more details on specific text language.

>>> Reply: We agree, and we have moved the phenotypic screening to the beginning of the results section. We also agree to de-emphasize the data of RsbL association with stressosome complexes. The appropriate changes have been made at lines 86 – 88, 432 – 434, and 510 - 517.

2. This reviewer suggests that the authors remove language from the manuscript that alludes to conclusions not shown by the data presented regarding RsbL clustering. For example, lines 94, 95, 544, 569, 570. In these instances, the authors state the clustering of RsbL is stressosome associated, yet no evidence is shown for that conclusion. Data showing RsbL fusion clustering in a stressosome deficient cell or other co-localization assays would give weight to those statements. Additionally, the fact that the fusion is nonfunctional for light sensing makes the clustering phenotype observed puzzling. Any affirmative conclusions about the RsbL fusion need further evidence and are not supported as they currently stand in the manuscript.

>>> Reply: We agree, and we have rephrased the text accordingly (Line 24 – 25, 86 – 88, 432 - 434, 522 - 524). The assumption that the clustering of RsbL might be stressosome-associated is based on the change in the diffusion coefficient. We acknowledge that it is unfortunate that RsbL fusions are no longer functional in light sensing, yet the clustering is still light-dependent.

3. Given that the localization (membrane bound vs free) and diffusion rates of RsbR1 change in the prli42 null strain, a complementary experiment would be to make the N-terminal mutant of prli42 known to prevent Prli42 interaction with RsbR1 and measure RsbR1 construct dynamics. This could refine the molecular model of interactions between the two proteins or if found differently propose new hypotheses.

>>> Reply: The suggested experiment is important to understand the interaction between Prli42 and RsbR1 and has been done by immunoprecipitation and immunoblotting in a previous study[5]. However, we found that the growth rates and acidic stress responses of the wild-type and *prli42* null strain are identical in the phenotypic screenings (See Phenotypic screening of the integrative strain, Supplementary Fig. 11, and Supplementary Fig. 12). Hence, Prli42 is important for tethering the stressosome to the membrane but does not play essential roles in sensing stress. The precise mode of action of Prli42 remains to be uncovered. Prli42

has been hypothesized that it could bring the stressosome into contact with a membrane protein, but that protein is as-yet-unidentified[5,6]. Therefore, the work on the complementary experiment is beyond the scope of our study.

4. One important question about stressosome structure is whether in vivo stressosomes are heterogenous complexes and whether the stoichiometry of the different RsbR paralogs changes during stress sensing and signaling. It seems that the RsbR1 fusion characterization was done in a wild type strain (unless I missed it) carrying the other RsbR paralogs, and therefore the RsbR1 localization involved a stressosome containing other RsbR members. Dessaux et al showed in 2020 that RsbR1 interactions are affected by the presence of its paralogs, what would happen to RsbR1 dynamics when it is the only the sensor in the stressosome? This experiment would add mechanistic insight to the phenomena observed by the authors and strengthen the impact the results have.

>>> Reply: We appreciate the suggestion of the reviewer, but we also feel that it is beyond the scope of this study.

Reviewer #3 (Remarks to the Author):

This manuscript by Tran et al. is both interesting and important, as it is at a key frontier for studies of signal sensation and transduction by bacterial stressosomes—namely, to visualize in vivo the molecular-level events that accompany stress sensation and signaling. This report takes a step towards the goal of understanding the dynamics of constituent proteins at the individual stressosome level to initiate the SigB-mediated stress response. In this work, the authors use single-particle tracking and other super-resolution fluorescence microscopic techniques to probe the localization and motion of two stressosome-associated sensors (RsbR1 and the light sensor RsbL) and one membrane-bound accessory factor (Prli42) that putatively tethers stressosome-associated factors to the cell membrane. The experiments are well done, and I am enthusiastic about the data.

Perhaps my biggest difficulty with this manuscript was that it does not follow a logical progression, making it very difficult to understand. What is the central question of the paper? There are many disparate results: from diffusion in different cell zones to localization of different proteins, many different diffusion numbers, plasmid-borne

and integrated genes, but what is the overall message? I understand that some of the findings are disparate, but for the sake of the reader the text needs to lead the reader logically from question to experiment to answer.

In my reading, the most important and exciting findings of the study are 1) confirmation that RsbR1 membrane-proximal location largely depends on Prli42; 2) that mEos-RsbR1 shows two different diffusion coefficients, suggestive of different association states; and 3) that mEos-RsbL diffuses faster than RsbR1 but clusters as cells are irradiated with light, slowing RsbL diffusion to values similar to that of RsbR1.

>>> Reply: We thank the reviewer for his/her favourable evaluation of the paper and valid suggestions. A number of his/her comments have been addressed in the rebuttal to R1 and R2.

Main concerns

1. It was unclear how some of the categories used in the paper were derived. Are the "membrane-bound" and "clustered" fractions of the fluorescent proteins defined by their diffusion coefficients or by their cellular localization? What were the criteria for placing a particular particle into each category?

>>> Reply: Very good point, thank you. We added a paragraph to explain the categories (Lines 280 - 290). SMdM and SPT provide both localization and mobility data. We categorised the protein states on the basis of individual trajectories of particles and the obtained diffusion coefficients; the latter gives an estimate of the size. However, it is practically impossible to determine the exact number of diffusive states on the basis of the tracking data[2]. Hence, we first used the localization data (membrane or cytoplasm; different conditions) and then the tracking (SPT) and SMdM data to estimate the diffusion coefficients, from which we infer whether or not the proteins cluster.

2. Because of its potential interaction with stressosomes, mEos-Prli42 diffusion is likely not representative of a generic membrane protein, and its diffusion is on the same order as the "mBd" and "Cl" fractions of R1 and L, consistent with an interaction. To test how Prli42-stressosome interactions impact the diffusion of each, a useful control would be a mutant of mEos-Prli42 that does not interact with RsbR1 (e.g., the R8A mutation; Impens et al. 2017).

>>> Reply: Membrane diffusion is largely determined by the viscosity of the membrane (see also Table 1 in Doeven et al. 2005)[7], and the diffusion coefficient of membrane-bound proteins has a different dependence on size than of water-soluble proteins (see Ramadurai et al (2009) JACS 131: 12650)[8]. The hydrophobicity properties of Prli42 make it a genuine membrane protein, and the diffusion coefficient of mEos3.2-Prli42 is in accordance with observations made for other membrane proteins[9–14]. Therefore, we feel that making the R8A mutation would not yield much additional information. (See comment 3 of Reviewer 2)

3. The finding that mEos-RsbR1 has two distinct diffusion coefficients that are several-fold different is quite intriguing, because it suggests two different association states. But the faster-diffusing population is still much slower (0.15-0.29 μm2/s) than free mEos and is not greatly impacted by the presence of Prli42, suggesting that neither association state is free—are we seeing single stressosomes and then stressosome clusters? How do the authors interpret this central finding of the study?

>>> Reply: We speculated that RsbR1 in the free (Fr) state is in the form of protomers of RsbR1-RsbS with D = 0.46 – 0.75 $\mu m^2/s$, whereas Rsb1 as part of the full stressosome complex (RsbR1-RsbS-RsbT) has D = 0.01 - 0.03 $\mu m^2/s$). We thank the reviewer for pointing out that the “free state” is confusing in the context of mEos3.2-RsbR1. We now refer to the component with D = 0.46 – 0.75 $\mu m^2/s$ as the Intermediate state (Int), whereas monomeric mEos3.2-RsbR1 is the free state. We have modified the text and figures accordingly.

We emphasise that when mEos3.2-RsbR1 is highly expressed from a plasmid (without in-frame fusion to the genes for RsbS and RsbT) the molecules end up in large immobile aggregates. This is the reason why subsequently we made chromosomal integration of *mEos3.2::rsbR1* in-frame with *rsbS* and *rsbT*. For mEos3.2-RsbR1 molecules at membrane-proximal locations (mBd fraction), we observe D = 0.12 – 0.29 $\mu m^2/s$, which is similar to the diffusion of Prli42 and suggests a fraction of monomeric mEos3.2-RsbR1 bound to Prli42 or unknown membrane components. We provide additional Supplementary Videos 1 and 2 to show the localization and clustering of RsbR1.

4. I am sympathetic to the intriguing idea that RsbL clustering might represent light-dependent association of L with stressosomes. But two difficulties with this

interpretation are 1) that the “Cl” fraction of RsbL diffuses faster than the “Cl” fraction of RsbR1 and 2) that R1 clustering is much rarer in the reconstructed images than L clustering after irradiation. If the clustering of L is into stressosomes, why don’t we see the same clustered pattern for R1? As this is another main finding of the paper, it deserves careful interpretation. It is certainly possible that the clustering is independent of stressosomes and is just a newly discovered property of RsbL after light exposure—perhaps even a mechanism by which the light response is turned off?

>>> Reply: The reviewer has partly given the answer him/herself. We added a paragraph (Lines 510 – 517) to discuss the clustering effect of RsbL. Indeed, RsbL forms clusters upon illumination, which is completely novel to the current knowledge. Unfortunately, we cannot conclude whether RsbL is associated with stressosome with the diffusion and localization data. However, since RsbL is one of the RsbR paralogs, it is likely that RsbL forms protomers with RsbS in a similar way to RsbR1-RsbS and form stressosome complexes. Therefore, there are two mutually exclusive hypotheses for the clustering of RsbL upon illumination: (1) RsbL is associated with stressosome complexes or (2) the clustering upon illumination could be independent of stressosomes - a newly discovered property for RsbL.

Minor points

1. The title is vague—it sounds like a review. It should instead reflect the central message of the paper (that’s up to the authors but should be specific). Two examples: “RsbR1 of Listeria monocytogenes displays two diffusion states and is membrane-localized by Prli42” or “Stressosome sensor proteins in Listeria monocytogenes display clustering and membrane localization”. Or even “Stressosome sensor proteins in Listeria monocytogenes show multiple diffusion states in vivo”.

>>> Reply: We have changed the title to “Super-resolving microscopy reveals the localizations and movement dynamics of stressosome proteins in *Listeria monocytogenes”.*

2. The abstract and text sometimes make claims that are not strictly supported by the data, e.g., l. 25-26 and 94-95. Association of RsbL with the stressosome complex

upon exposure to light is not shown here. The diffusion rates of clustered L are perhaps consistent with stressosome interaction, but there are other possible interpretations. Another small example is l. 422-423, that tagging of RsbL hampers its light sensing. It seems clear that it hampers signaling to sigB, but the light-dependent clustering result suggests that it can still indeed respond to (and thus sense) light.

>>> Reply: We rephrased the text to make the points clearer.

3. Proper genetic nomenclature should be followed in the text and strain table ("::" means an insertion).

>>> Reply: This is a valid point. We corrected the genetic nomenclatures in the text, images, and tables.

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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addressed my questions and the manuscript was improved. I recommend publication.

Reviewer #2 (Remarks to the Author):

In this revised manuscript by Tran et al., the authors have done a good job addressing many of the reviewer comments. The data that this paper contributes to the field are valuable, despite the fact that not all of the observed phenomena are yet fully explained or connected to other work on the stressosome. In the revision, the interpretations match the data rather than drawing inferences that are not formally supported by the results. The authors should be applauded for undertaking this study, which represents a very substantial amount of careful microscopic analysis, not to mention the genetic work. It will be interesting to gain in future studies a more complete cell biological picture of stressosome dynamics within cells. In my opinion, the paper is essentially publication ready. I have only a few minor textual recommendations.

Minor points

ll. 292, 440: Agreed that RsbR1 has three distinguishable diffusive states, but whether each diffusive state represents a biologically relevant (i.e., functionally distinct), state formally remains unknown, as there are no functional tests for each state nor phenotypic differences that are conditioned on a particular diffusive state. Thus I advocate that these statements be qualified ("…diffusive states that may correspond to biologically relevant differences…", for example).

ll. 495-6, 506, 516-517, 524. In the interpretations regarding the interesting light-stimulated clustering of mEos3.2-rsbL, caution should be used to avoid drawing conclusions about the native function of RsbL (e.g., that light-sensing is a 2-step process that includes clustering). The data indicate only that mEos3.2-RsbL, which is nonfunctional based on the ring-formation phenotype, clusters. Hence it remains unknown whether clustering is a property of unlabeled RsbL and whether, if it occurs, whether it has a biological function or is incidental.