nature portfolio

Peer Review File

Filamentation-driven peripheral clustering of the inducible lysine decarboxylase is crucial for E. coli acid stress response

Corresponding Author: Dr Irina Gutsche

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, the authors investigate the role of LdcI polymerization in E. coli acid stress response. Using a polymerization-deficient mutant, they conclude that LdcI filamentation is required for its peripheral clustering and that this clustering is essential for acid stress resistance. While the work is innovative and technically rigorous, several issues require clarification to fully support the central claims.
1. The study lacks direct evidence for LdcI polymerization in vivo. Since polymerization is central to the authors' model, additional support for polymerization status in vivo would strengthen the claim. It remains unproven whether the polymerization-deficient mutant is fully incapable of forming filaments in the more complex intracellular environment. The residual clustering observed in the mutant is interpreted by the authors as possibly arising from decamer stacking, but this interpretation is speculative and not experimentally validated. It is equally plausible that the mutant retains partial filament-forming ability.
2. Line 286. Since the triple E445A-D447A-R468A mutation of E. coli LdcI abolishes decamer stacking in vitro, we engineered a mutant 3M strain harboring these three mutations in the cadA gene. And line 84, we have engineered a structure-based triple triple mutant of E. coli LdcI that loses polymerisation capacity in vitro and remains decameric even at low pH. And line 366. Curiously, the 3M LdcI strain still exhibited intracellular clustering despite its impaired polymerisation capacity, observed in vitro at low protein concentrations. This suggests the mutant decamer may retain residual stacking ability in vivo. I have no idea whether the 3M strain could form decamer in vivo or in vitro because there is no figure or reference to prove. Also, the authors should clarify the polymerization of decamers, the polymerization of stacking, and the polymerization that leads to the formation of filament, to avoid confusion.
3. Discussion line 432. The claim that "we have clearly established that LdcI clustering is driven by its polymerization" requires stronger evidence. The 3M mutant is still capable of forming clusters, albeit in reduced number and size, suggests that clustering may occur independently of full polymer formation. The authors should either temper this statement or provide additional explanation.
4. The Western blot shown in the supplementary material lacks a visible loading control. For reliable interpretation of protein levels, an internal control should be clearly presented (or the evidence of the same sample loading amount).
5. The growth phenotype of the polymerisation-deficient mutant is tested only under acid stress (pH 4.6). Growth data under neutral pH or non-stress conditions should be included to demonstrate that the observed defect is specific to acid stress and not due to general growth impairment.
6. Line 44. Lysine import by LysP under acidic conditions releases…Grammatical error.
7. Line 50. until CadC inhibition by excess of the exported cadaverine shuts down the system… Grammatical error.
8. Line 83 triple triple mutant. Check this.
9. Figure 1b wasn't been cited by any results or discussion.
10. Line 273. In addition, the resulting cluster distributions do not corroborate our initial visual impression- based on a limited 3D dSTORM dataset and lacking the present quantitative assessment- of a potential long-range pattern in cluster organisation. Is the reason for the inability to confirm due to a lack of quantitative assessment? Please revise the expression to make it easier to understand.
11. Line 274. The en dash should be replaced by the em dash. There are also similar errors in the following text.

Reviewer #2

(Remarks to the Author)
The manuscript discusses the role of inducible lysine decarboxylase (LdcI) filamentation in Escherichia coli's acid stress response. In a previous work, the authors highlighted its importance in enzyme clustering and activity optimization under acidic conditions.

The authors investigate herein the physiological relevance of LdcI filamentation using advanced microscopy techniques:

1) The dSTORM super-resolution technique was utilized to capture high-resolution images of LdcI localization.

2) The study establishes a semi-automated workflow for detecting and analyzing LdcI clusters quantitatively. Clustering analysis was performed using density-based methods. The method allows for estimating cluster volume and identifying their relative location within the cell—particularly whether they lie closer to the periphery or are more centrally distributed.

3) LdcI (WT) polymerizes into filaments at acidic pH, forming clusters that are predominantly located at the cell periphery, without clear polar localization.

4) A mutant strain (3M) of E. coli lacking LdcI polymerization shows reduced cluster size and impaired acid stress response. The average cluster volume is significantly reduced (by ~2.6-fold), although peripheral clustering is still observed. In addition, the 3M strain shows weaker acid stress resilience, indicated by delayed growth and impaired pH buffering.

These results support the hypothesis that LdcI filamentation enhances the acid stress response by facilitating larger peripheral clusters, that may be more functional.

I find the study thorough and convincing in its conclusions. However, I have a few specific questions that need to be addressed, and suggestions for clarification.

1) In this study, the authors use 3D-STORM super-resolution microscopy. The use of such a state-of-the-art technique is crucial due to the high density and heterogeneity of the protein signal, which conventional microscopy cannot resolve adequately. I suggest highlighting the need for this technique earlier in the main text, as it's critical for understanding the data quality and the clustering analysis. Also, what is the spatial resolution achieved?

2) Mutant rationale: Could the authors provide more explanation (and also earlier in the text) about the structural basis for expecting the 3M mutations to prevent filamentation in vivo?

3) Distribution under stress: Is it possible to analyze how LdcI cluster distribution changes dynamically during stress? Even with a strong global signal, some insight (qualitative or quantitative) into temporal redistribution could strengthen the physiological interpretation.

4) Clustering variability: The manuscript mentions significant variation in signal density across cells. Could the authors expand on how this was accounted for statistically, and whether the results remain robust across the full population?

5) Thresholding in segmentation: In Figure 3b, it appears that some clusters visible in the super-resolution images are not detected in the lower segmentation panel. Could the authors clarify how the clustering thresholds were chosen, and whether results like those in Figure 3a remain significant under more permissive thresholds?

6) The authors mention that pairs of recently divided cells could not be accurately separated by DBSCAN, as the largest clusters detected before full cell segmentation often spanned both daughter cells. However, is there no way to improve this analysis? It seems that commonly used software in the community, such as CellProfiler, could facilitate this task. For example, dividing cells could first be segmented individually using one of the many available algorithms, and then LdcI could be segmented as secondary objects.

7) The extremely low p-value in Figure 3a seems surprising given the apparent overlap in distributions. Could the authors confirm whether this value is correct?

8) Some aspects, such as the clustering algorithm choice and implementation, could benefit from clearer explanation for readers not specialized in super-resolution microscopy.

Finally, a few minor editorial notes:
• Line 136: delete "of cells"
• Line 292: correct to "Supplementary"
• Figure 2 legend: use "b: Gallery of..." instead of the current phrasing

Reviewer #3

(Remarks to the Author)
Dear authors,

Please find below my comments for your manuscript.

The manuscript "Filamentation-driven peripheral clustering of the inducible lysine decarboxylase is crucial for E. coli acid stress response" by Kirchner and colleagues addresses a possible novel regulatory mechanism of the enzyme LdcI through oligomerisation into filamentous structures in E. coli exposed to an acidic pH environment. The authors applied 3D Single Molecule Localisation Microscopy to visualise LdcI clusters in parental E. coli MG1655 and in the 3M mutant, which carries three site-directed mutations in the ldcI gene that result in a variant unable to polymerise under acidic pH. The authors carefully inserted the mutated gene into the same locus as the wild-type ldcI to ensure that the differences in filament formation were not due to differences in expression levels between the wild-type and the mutant. Indeed, the authors showed by western blotting that expression levels are similar between the wild-type and the 3M mutant. Furthermore, they demonstrated that the mutant is impaired in its adaptation to acid stress but can still recover growth and raise the medium's pH. Microscopy analysis also revealed that the 3M mutant does not form the same cluster pattern observed in the wild-type.

Although I am not a specialist in 3D Single Molecule Localisation Microscopy, I have made some comments regarding the functional aspect of the findings, which I would like the authors to consider when revising the manuscript.

Specific comments:
1. Lines 10–12: I suggest the authors write this sentence in the past tense: "We established..., and demonstrated...".

2. Line 14: I suggest the authors complete the sentence with "Rather than LdcI intracellular locality" or a similar phrase.

3. Lines 17–19: The authors state that the filamentous oligomeric form of LdcI contributes to E. coli buffering via a spatial optimisation of enzymatic activity. What does this mean? Do the filaments localise in a cytoplasmic region with a higher concentration of lysine to supply LdcI? Or does LdcI polymerisation into filaments increase its activity?

4. Line 41: I believe the correct is cad italicised.

5. Line 50: Correct the typos. "antiporter".

6. Line 83: Two "triple" words are in sequence.

7. Line 103: What is the reference for pJL72? Into which restriction sites did the authors clone the mutated gene? What are the positions of ldcI-3M and the KmR cassette in the PCR product used for Lambda/Red recombineering? Please provide details of the Lambda/Red protocol used, such as the mass of PCR product in ng, the procedure for mutant selection, and any parameters that differ from the original reference [22].

8. Line 105: Please replace "kanamycine" with "kanamycin".

9. Line 108: The correct name is pCP20.

10. Line 113: Did the authors use orbital shaking for the E. coli cultures? Were Erlenmeyer flasks used? What was the flask volume? What shaking speed (rpm) was used?

11. Line 134: Did the authors purchase the STORM buffer and GLOX solution? If so, please provide the manufacturers. If not, please provide the composition and any relevant protocol details needed to replicate the experiments.

12. Line 135: What 1.5% LMP agarose volume was pipetted onto the bacterial suspension?

13. Line 136: Please remove "of cells".

14. Line 223: Could the authors use a nanobody against a constitutively expressed protein in E. coli to monitor the percentage of permeabilised cells?

15. Figure 2 legend: The panel labelled "c" should be "b".

16. Line 285: Please correct the spelling; the correct term is Enterobacteriaceae.

17. Supplementary Figure 3: Please complete the y-axis of the graph "a" with "OD600".

18. Line 278: I disagree with the title of this subsection. I would not use the term "impedes", as the 3M mutant partially recovers from acid pH stress. It is clearly not growing at the same rate as the wild type, but it is still growing and increasing the extracellular pH. A more appropriate title would be: "Disrupting LdcI polymerisation delays E. coli acid stress response".

19. Line 363: Have the authors considered whether the reduced activity in the LdcI 3M triple mutant might be due to the amino acid substitutions themselves rather than the loss of self-polymerisation? Has the activity of both wild-type and 3M mutant LdcI been determined in vitro? Whether the lack of LdcI polymerisation could affect cadaverine exportation via CadB.

20. References: Please revise the reference list. I identified spelling mistakes and scientific names that are not italicised.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript and the rebuttal letter, the previously raised review comments have been appropriately addressed or corrected.

Reviewer #2

(Remarks to the Author)
I have reviewed the revised version of the manuscript and I am pleased to confirm that the authors have adequately addressed all of my previous concerns. The revisions have strengthened the manuscript, and I now find it suitable for publication.

Reviewer #3

(Remarks to the Author)
Dear Authors,
Dear Editor,

I have carefully reviewed the revised manuscript entitled “Filamentation-driven peripheral clustering of the inducible lysine decarboxylase is crucial for E. coli acid stress response”, submitted to Communications Biology.

The authors have comprehensively addressed the comments raised in the previous round of review, including all the points I raised as Reviewer 3. I congratulate the authors on the significant improvements they have made to the manuscript in response to the feedback. To make it easier for the Editor to identify the points, I have summarised them below:

1. The authors have corrected all typographical and formatting issues, including gene and species name formatting, figure labels, and textual clarity.

2. The Materials and Methods section has been extensively revised to provide the necessary details on strain construction, plasmids, buffer compositions, and culture conditions, ensuring full reproducibility.

3. The points regarding clarity of interpretation, particularly the meaning of “spatial optimisation” about LdcI polymerisation, have been adequately expanded in the revised Discussion section.

4. Scientific concerns regarding whether the mutant phenotypes could result from effects beyond polymerisation were thoughtfully addressed. The authors provided additional context based on preliminary ITC data supporting that the enzymatic activity of the triple mutant is not significantly affected by the amino acid substitutions themselves.

5. The suggestion regarding the use of a nanobody-based permeabilisation control was acknowledged. Although not experimentally implemented in the current study, the authors provided a reasonable justification, referencing prior optimisation published in Jessop et al. (PNAS, 2021).

Yours sincerely,
Marcelo Müller-Santos

Point-by-point response to the reviewers

Reviewer 1:

Comment 1:
The study lacks direct evidence for LdcI polymerization in vivo. Since polymerization is central to the authors' model, additional support for polymerization status in vivo would strengthen the claim. It remains unproven whether the polymerization-deficient mutant is fully incapable of forming filaments in the more complex intracellular environment. The residual clustering observed in the mutant is interpreted by the authors as possibly arising from decamer stacking, but this interpretation is speculative and not experimentally validated. It is equally plausible that the mutant retains partial filament-forming ability.

Answer:
We thank the reviewer for the comment. We would first like to emphasise that, as in most papers on polymerisation of dihedrally symmetric enzymes by linear stacking into filaments, and after having ourselves solved the cryo-EM structures of *E. coli* LdcI and *P. stuartii* Adc both as individual D5-symmetric barrels and as polymers formed by linear stacking of decamers (Jessop et al., PNAS 2021; Jessop et al., Comms Biol 2022), for these kinds of filaments the terms polymerisation, stacking and filamentation are commonly used as synonyms. We now make this point clearer in the third paragraph of the introduction. We would also like to point to the citation of the reference of Hvorecny and Kollman (COSB 2023, reference 42 in the manuscript), which is specifically dedicated to this matter. In the case of LdcI, only the D5-symmetric form has been observed to stack into linear polymers *in vitro*, which we designate as stacks, polymers and filaments in our previous work (Jessop et al., PNAS 2021). Consequently, LdcI stacks, filaments and polymers are equivalent to each other. We therefore agree with the reviewer that the residual clustering might be due to LdcI retaining partial filament-forming ability, as was already noted in the original version of the manuscript, in the discussion section.

Comment 2:
Line 286. Since the triple E445A-D447A-R468A mutation of E. coli LdcI abolishes decamer stacking in vitro, we engineered a mutant 3M strain harboring these three mutations in the cadA gene. And line 84, we have engineered a structure-based triple triple mutant of E. coli LdcI that loses polymerisation capacity in vitro and remains decameric even at low pH. And line 366. Curiously, the 3M LdcI strain still exhibited intracellular clustering despite its impaired polymerisation capacity, observed in vitro at low protein concentrations. This suggests the mutant decamer may retain residual stacking ability in vivo. I have no idea whether the 3M strain could form decamer in vivo or in vitro because there is no figure or reference to prove. Also, the authors should clarify the polymerization of decamers, the polymerization of stacking, and the polymerization that leads to the formation of filament, to avoid confusion.

Answer:
We thank the reviewer for pointing out that the use of the terms stacking, polymerisation and filamentation in the original version of the manuscript may have appeared confusing. We hope that the modified third paragraph of the introduction, as cited in the answer to the comment 1, addresses this point satisfactorily. Regarding the residual stacking ability of the 3M mutant, we would like to refer to the main text and the Supplementary Figures 7 and 8 of our Jessop et al., PNAS 2021 manuscript (reference 17 in the current manuscript) and to the answer to the comment 1 above. Indeed, our previous work shows that *in vitro*, at pH 5.7, optimal for LdcI activity, this mutant remains in a decameric, non-stacked form. As the reviewer mentioned, the residual clustering suggests some residual stacking ability *in vivo* that is not present *in vitro*, at least at the concentrations used for the negative stain EM analysis. This point is addressed in

the manuscript discussion section. In addition, and also in order to satisfy the comments of the reviewer 3, we have now completely rewritten the materials and methods part corresponding to the selection and construction of this mutant. We now start this part with an explicit explanation of the choice of these mutations based on our previous negative stain EM analysis, extensively described in the Jessop et al., PNAS 2021 paper.

Comment 3:
Discussion line 432. The claim that "we have clearly established that LdcI clustering is driven by its polymerization" requires stronger evidence. The 3M mutant is still capable of forming clusters, albeit in reduced number and size, suggests that clustering may occur independently of full polymer formation. The authors should either temper this statement or provide additional explanation.

Answer:
The sentence has now been tempered.

Comment 4:
The Western blot shown in the supplementary material lacks a visible loading control. For reliable interpretation of protein levels, an internal control should be clearly presented (or the evidence of the same sample loading amount).

Answer:
The 'cell culture' section of the Materials and Methods states that the number of cells equivalent to an OD 1 suspension in 1 ml was used for all samples. We have now repeated one of the blots because of the absence of a purified LdcI protein band on the previous version. This band can now be seen in the middle line under 3M in Supplementary figure 4c, and the panel has been reorganised to include the purified protein bands for size indication. The graph in supplementary figure 4d has been changed accordingly. All full, non-cropped images of the Western blots are now available in the Supplementary figure 5. We thank the reviewer for pointing out this inconsistency.

Comment 5:
The growth phenotype of the polymerisation-deficient mutant is tested only under acid stress (pH 4.6). Growth data under neutral pH or non-stress conditions should be included to demonstrate that the observed defect is specific to acid stress and not due to general growth impairment.

Answer:
We agree that these growth curves should be included. Indeed, before the cells were exposed to acid stress for our pH-shift experiments, they were all grown to the same starting OD of 0.1. We collected the growth data from these cultures and compiled a new graph showing no difference in growth under pH 7 between the WT and 3M strains. This information is now included as Supplementary figure 3 and the relevant values have been added to the Supplementary file 1.

Comment 6:
Line 44. Lysine import by LysP under acidic conditions releases… Grammatical error.

Answer :
The structure of the sentence has been simplified.

Comment 7:
Line 50. until CadC inhibition by excess of the exported cadaverine shuts down the system… Grammatical error.

Answer:
We thank the reviewer for the comment and simplify the sentence.

Comment 8:
Line 83 triple triple mutant. Check this.

Answer:
The second triple has been removed.

Comment 9:
Figure 1b wasn't been cited by any results or discussion.

Answer:
We thank the reviewer for pointing this out. The reference to Figure 1a has been extended to all of Figure 1.

Comment 10:
Line 273. In addition, the resulting cluster distributions do not corroborate our initial visual impression- based on a limited 3D dSTORM dataset and lacking the present quantitative assessment- of a potential long-range pattern in cluster organisation. Is the reason for the inability to confirm due to a lack of quantitative assessment? Please revise the expression to make it easier to understand.

Answer:
As mentioned in the present manuscript and Jessop et al. [ref 17 of the manuscript], our previous hypothesis about an existence of a potential long-ranged order in the cluster organisation was based on a purely visual inspection of a very small 3D dSTORM dataset. Using the rigorous quantitative approach presented in the current manuscript, we show that this initial hypothesis was not correct. Consequently, and because this hypothesis was only briefly mentioned at the end of the previous manuscript, we don't think that it is necessary to explain it in more details.

Comment 11:
Line 274. The en dash should be replaced by the em dash. There are also similar errors in the following text.

Answer:
The relevant dashes have been replaced.

Reviewer 2:

Comment 1:
In this study, the authors use 3D-STORM super-resolution microscopy. The use of such a state-of-the-art technique is crucial due to the high density and heterogeneity of the protein signal, which conventional microscopy cannot resolve adequately. I suggest highlighting the need for this technique earlier in the main text, as it's critical for understanding the data quality and the clustering analysis.

Also, what is the spatial resolution achieved?

Answer:
We thank the reviewer for pointing out that the manuscript could benefit from an earlier introduction into the methodology. We now mention the usage of 3D dSTORM in the introduction to highlight the critical nature of the chosen technique for the manuscript's conclusions.
In terms of spatial resolution, we now report the average uncertainty value in xy (and z) in the corresponding Methods section. Specifically, across all unfiltered table files the average xy-uncertainty of a localisation was found to be 24 ± 7 nm. For determination of the axial resolution attainable by the used microscope setup, we carried out an additional stream recording of a sample of planar fluorescent beads and averaged the z-positions of the detected beads, resulting in an approximate precision of 20 nm.

Comment 2:
Mutant rationale: Could the authors provide more explanation (and also earlier in the text) about the structural basis for expecting the 3M mutations to prevent filamentation in vivo?

Answer:
We thank the reviewer for pointing out that the rationale for the design of the 3M might not be completely clear to the reader until reaching the corresponding results section of the manuscript. The rationale for the mutant design was described in Jessop et al., PNAS 2021 [reference 17], where the structure of the LdcI stack was solved using single particle cryo-EM and the amino acids involved in the stacking interfaces were identified. As can be seen in supplementary figures 7 and 8 of the Jessop et al. manuscript, this information was used to design several different mutant variants of LdcI, which were expressed and purified as the WT enzyme. Some of these purified mutants, including the triple mutant used in the present study, were observed by negative stain electron microscopy to remain decameric *in vitro,* even under pH conditions optimal for polymerisation of WT LdcI into filamentous stacks. Based on this finding, here we chose to construct and use the 3M MG1655 strain for phenotypic analysis and fluorescence imaging. This rationale is now described in the corresponding section of the Methods.

Comment 3:
Distribution under stress: Is it possible to analyze how LdcI cluster distribution changes dynamically during stress? Even with a strong global signal, some insight (qualitative or quantitative) into temporal redistribution could strengthen the physiological interpretation.

Answer:
An investigation of time-dependent dynamics would indeed be a possible next step in the investigation of LdcI spatial distribution under acid stress. The reason for the usage of 90 min as the time point of observation here is presented in [17] in supplementary figure 3, where we showed that LdcI protein levels are highest after 1-2 h of exposure to acid stress. We thank the reviewer for again pointing out the importance of continuing to investigate the dynamics of this process, but feel that adding it to the current manuscript would extend its scope too much. Nonetheless, we have added a mention of the importance of dynamics in the discussion and a in the beginning of the relevant results section.

Comments 4 and 5:
Clustering variability: The manuscript mentions significant variation in signal density across cells. Could the authors expand on how this was accounted for statistically, and whether the results remain robust across the full population?

and

Thresholding in segmentation: In Figure 3b, it appears that some clusters visible in the super-resolution images are not detected in the lower segmentation panel. Could the authors clarify how the clustering thresholds were chosen, and whether results like those in Figure 3a remain significant under more permissive thresholds?

Answer:
As stated in the manuscript, the variability of signal density across the cells arises most probably from the population-wide regulation of acid stress resistance as presented in Brameyer et al. [34], although we cannot completely rule out a possibility of a heterogeneous permeabilisation of the cells. Evidence of efficient permeabilization upon following our experimental procedure, is also presented in supplementary figure 3 of our previous manuscript [17]. Only cells which could be segmented from the full recorded volume as a single cluster recognised by DBSCAN were used for further investigation of intracellular cluster size. In contrast to the identification of intracellular clusters with FOCAL3D, this segmentation procedure was based on visual assessment and is illustrated in supplementary figure 1. After segmentation, the single cells are used as input for FOCAL3D. The significant difference between these two algorithms is the feature of FOCAL3D that uses subvolumes populated by the data points for clustering, instead of using radial distances between individual points. The exact procedure for setting the thresholds of subvolume size, point density in one subvolume, and number of neighbouring subvolumes necessary to form a cluster is determined in a screening procedure which we performed for every cell individually, as described in detail in the original FOCAL3D publication [32]. To make this clearer, we have now added additional explanations to the Methods, as well as to the Results section.
We would like to emphasize that such tuning of clustering parameters is much less based on human intervention than setting DBSCAN parameters based on visual inspection. Thus, we decided to use FOCAL3D to account for the variability in signal across different cells (since the clustering parameters can be changed for each cell individually). As the reviewer points out, this sometimes resulted in clustering that appeared counter-intuitive upon initial visual inspection. This was however not only observed for the 3M but also the WT. We have incorporated example of these observations in both Figure 2 (most clearly in 3rd cell from the right) and 3 (most clearly in 1st cell from the right) to show that this is not a phenomenon that concerns the 3M mutation only. More permissive thresholds for the clustering parameters would certainly increase their size as the subvolumes determined by FOCAL3D would increase in size. The uncertainty of which density thresholds should exactly be taken is the central reason why we opted for FOCAL3D to not only detect the clusters, but also determine the optimal search parameters based on the included parameter-tuning procedure.

Comment 6:
The authors mention that pairs of recently divided cells could not be accurately separated by DBSCAN, as the largest clusters detected before full cell segmentation often spanned both daughter cells. However, is there no way to improve this analysis? It seems that commonly used software in the community, such as CellProfiler, could facilitate this task. For example, dividing cells could first be segmented individually using one of the many available algorithms, and then LdcI could be segmented as secondary objects.

Answer:
We thank the reviewer for commenting on this important point. We agree that other software may have also been used. In the present work, we used data obtained from pairs of recently divided cells for determination of cluster size and the comparison of cluster size between the different *E. coli* variants. However, these cell pairs were excluded from the procedure to determine distance ratios of the clusters in order to prevent effects of overly-complex geometries of the cell hull on the calculations.

Comment 7:
The extremely low p-value in Figure 3a seems surprising given the apparent overlap in distributions. Could the authors confirm whether this value is correct?

Answer:
A recalculation of the value gave the same value again. We originally used the 'asymptotic' mode of the scipy.stats.mannwhitneyu function due to computational restrictions, but reran the calculations with the 'exact' mode enabled 50 times with 300 randomly chosen pairs of values (WT vs. 3M) each. This returned an average *p*-value of 1.5×10^{-8} which is still exceedingly small and is now reported in the legend of Figure 3.

Minor editorial notes:
•*Line 136: delete "of cells"*
•*Line 292: correct to "Supplementary"*
•*Figure 2 legend: use "b: Gallery of…" instead of the current phrasing*

Answer:
These and other typos have been corrected.

Reviewer 3:

Comment 1:
Lines 10–12: I suggest the authors write this sentence in the past tense: "We established..., and demonstrated...".

Asnwer:
This and the following sentences are now in past tense.

Comment 2:
I suggest the authors complete the sentence with "Rather than LdcI intracellular locality" or a similar phrase.

Answer:
We thank the reviewer for the suggestion and have added the clause to the end of the sentence.

Comment 3:
The authors state that the filamentous oligomeric form of LdcI contributes to E. coli buffering via a spatial optimisation of enzymatic activity. What does this mean? Do the filaments localise in a cytoplasmic region with a higher concentration of lysine to supply LdcI? Or does LdcI polymerisation into filaments increase its activity?

Answer:
We thank the reviewer for pointing out the ambivalence of this statement. This point is addressed in the second half of the first paragraph of the Discussion section. LdcI filamentation appears to have a strong impact on the cells ability to counteract acid stress as shown in supplementary figure 3.

Comment 4:
I believe the correct is cad italicised.

Answer:
We have now paid closer attention to italics.

Comment 5:
Line 50: Correct the typos. "antiporter".

Answer:
The spelling was corrected.

Comment 6:
Line 83: Two "triple" words are in sequence.

Answer:
The second instance of triple has been removed.

Comment 7:
What is the reference for pJL72? Into which restriction sites did the authors clone the mutated gene? What are the positions of ldcI-3M and the KmR cassette in the PCR product used for Lambda/Red recombineering? Please provide details of the Lambda/Red protocol used, such as the mass of PCR product in ng, the procedure for mutant selection, and any parameters that differ from the original reference [22].

Answer:
We apologize for the poor quality of the original version of this section of Materials and Methods and the supplementary table that accompanied it. We have now completely rewritten the paragraph describing the construction of the mutant strain and updated the table with the requested references. This full description now allows the reader to fully understand and reproduce the mutant strain construction. The details of the routine protocol for the recombination in *E. coli* using the Lambda/Red system are exactly the same as in the original Datsenko and Wanner, 2000 paper.

Comment 8:
Line 105: Please replace "kanamycine" with "kanamycin".

Answer:
The spelling has been corrected.

Comment 9:
Line 108: The correct name is pCP20.

Answer:
The spelling has been corrected.

Comment 10:
Line 113: Did the authors use orbital shaking for the E. coli cultures? Were Erlenmeyer flasks used? What was the flask volume? What shaking speed (rpm) was used?

Answer:
The cultures were grown in 2 stages. First, 2 L Erlenmeyers were used for pre-culturing at pH 7 under shaking at 180 rpm. Later, cells were transferred into 50 mL tubes (one per time point to measure) under acid stress and high lysine availability. These details are now added to the relevant Materials and Methods section.

Comment 11:
Line 134: Did the authors purchase the STORM buffer and GLOX solution? If so, please provide the manufacturers. If not, please provide the composition and any relevant protocol details needed to replicate the experiments.

Answer:
The solutions were prepared in-house and the Glucose Oxidase and Catalase enzymes were purchased from Sigma Aldrich. We followed a recipe presented in the reference 23. The composition and provider of the enzymes has been added to the relevant section of the Materials and Methods.

Comment 12:
Line 135: What 1.5% LMP agarose volume was pipetted onto the bacterial suspension?

Answer:
The agarose is initially dissolved in the STORM buffer, to which Cysteamine and GLOX are added. This final mixture is then put between slide and coverslip (on which the cell suspension was deposited before). A mention of this procedure was added to the relevant section of the Materials and Methods.

Comment 13:
Line 136: Please remove "of cells".

Answer:
The passage was removed.

Comment 14:
Line 223: Could the authors use a nanobody against a constitutively expressed protein in E. coli to monitor the percentage of permeabilised cells?

Answer:
We thank the reviewer for this suggestion. While this could indeed have been possible, we based the present study on our initial experiments assessing and optimising the *E. coli* cell permeabilisation as described in our previous work (Jessop et al., PNAS 2021).

Comment 15:
Figure 2 legend: The panel labelled "c" should be "b".

Answer:
The mistake has been corrected.

Comment 16:
Line 285: Please correct the spelling; the correct term is Enterobacteriaceae.

Answer:
The spelling has been updated.

Comment 17:
Supplementary Figure 3: Please complete the y-axis of the graph "a" with "OD600".

Answer:
The axis labelling was changed to OD600.

Comment 18:
Line 278: I disagree with the title of this subsection. I would not use the term "impedes", as the 3M mutant partially recovers from acid pH stress. It is clearly not growing at the same rate as the wild type, but it is still growing and increasing the extracellular pH. A more appropriate title would be: "Disrupting LdcI polymerisation delays E. coli acid stress response".

Answer:
We thank the reviewer for this suggestion, which we completely agree with. The title of the section has been changed accordingly.

Comment 19:
Line 363: Have the authors considered whether the reduced activity in the LdcI 3M triple mutant might be due to the amino acid substitutions themselves rather than the loss of self-polymerisation? Has the activity of both wild-type and 3M mutant LdcI been determined in vitro? Whether the lack of LdcI polymerisation could affect cadaverine exportation via CadB.

Answer:
We thank the reviewer for pointing this out. We did consider the possibility of the substitutions having an influence on the activity and performed preliminary ITC experiments to evaluate it. The K_M and k_{cat} values retrieved from these preliminary experiments for 3M and WT were very similar (317 µM vs 442 µM, 111/s vs 108/s). However, the concentrations used for *in vitro* activity measurements such as ITC are lower than those in the cellular environment at which the residual stacking of the LdcI decamers takes place. In addition, as shown by negative stain EM (Jessop et al., 2021), in contrast to the WT protein, the 3M mutant remains entirely decameric. Therefore, we decided not to include these ITC data in the manuscript but to focus on the effect of the polymerisation on cell growth and pH buffering. As for a potential direct effect of the 3M mutation on CadB, we did not perform these experiments in the frame of this study, which is centered on LdcI polymerisation. The activity of CadB in the 3M strain could be the subject of further investigations.

Comment 20:
References: Please revise the reference list. I identified spelling mistakes and scientific names that are not italicised.

Answer:
The references have been revised.