

Streptomyces use both polar and dispersed cell wall synthesis during exploratory growth

Corresponding Author: Dr Marie Elliot

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this article, the authors investigated the contribution of mreB to lateral cell wall synthesis in *S. venezuelae*, particularly its role in exploration, a peculiar feature of *Streptomyces* growth. The main conclusion and original finding is that *S. venezuelae* utilizes both polar and lateral expansion during this mode of growth. One of the conclusion of this work is that mreB1 is necessary for exploration. While the role of the mreB operon in exploration is convincing and very interesting, the claim that mreB1 alone is responsible for this phenotype is not fully supported by the experiments presented in the paper.

The peptidoglycan analyses should be better described to convincingly demonstrate the structural changes associated with exploration and the deletion of the mreB operon. In Figure 1, the representation of the L,D-crosslink is incorrect (D-Ala4 should be absent in the donor peptide stem). The peptidoglycan analyses are not sufficiently detailed to support the conclusions: (i) the methods section should include MS and MS/MS; (ii) the abbreviations for mucopeptides in the dataset are not fully explained. Does "G" stand for glycine, GlcNAc, or both? Which mucopeptides were identified as 3-3 or 4-3 crosslinks? How was glycine in the side chain distinguished from glycine at the C-terminus of peptide stems? I suggest including representative chromatograms to strengthen the conclusions.

The deletion of mreB1 leads to a growth defect on YP medium. The authors attribute this defect solely to mreB1 deletion, but complementation does not seem to fully restore the wild-type phenotype. The text states: "Introducing a wild-type copy of mreB1 at a heterologous site in the chromosome restored a more wild-type-like wrinkling pattern and alleviated the apparent cell lysis, although colonies remained smaller than wild-type." As shown, the phenotype of the strain lacking the entire mre operon does not seem to differ from that of the Δ mreB1 strain. Therefore, the mreB1 mutant phenotype might be due to a polar effect on downstream genes in the mreB operon. A markerless in-frame deletion of mreB1 (similar to what was done for the mreB2 mutant) would greatly support the conclusions. In Figure 2, the Δ mreB1::mreB1 strain producing mNeonGreen is missing, which raises further doubts about the specific contribution of mreB1. This control should be included. Exploration growth is convincingly affected by the deletion of the mre operon, but attributing the phenotype to a specific gene remains unsupported. The increase of D,D-crosslinks in the exploration mode and the loss of TDL labeling in the mreB mutant suggest an important role for the bPBP encoded by the mreB operon in this mode of growth. If this is the case, fast-growing hyphae would require a fast-acting enzyme (the bPBP) to sustain rapid growth, rather than a slower transpeptidase (an L,D-transpeptidase). This phenomenon would be distinct from the peripheral growth of *Mycobacteria*, which relies on the maturation of PBP-mediated peptidoglycan polymerization by L,D-transpeptidases (Elife, 2018, Oct 16:7.doi: 10.7554/eLife.37516). Regarding Figure 4, what is the labeling profile of an 11-day-old culture? If labeling occurs, this could suggest that other PBPs partially compensate for the loss of function of the bPBP.

In the introduction: "DivIVA is broadly conserved among Gram-positive bacteria (Hammond, White & Eswara, 2019), but its role in cell wall synthesis is confined to the actinobacteria, where it is an essential protein (Flärdh, 2003)." This sentence is confusing, as DivIVA is also involved in cell wall synthesis in other bacteria (e.g., streptococci).

"These glycan strands are joined via their NAM-affixed peptide stems, with these stems in turn being linked together by either D,D-crosslinks catalyzed by penicillin-binding proteins (PBPs) or L,D-crosslinks catalyzed by L,D-transpeptidases (Glauner, Höltje & Schwarz, 1988)." The formation of crosslinks is catalyzed, rather than the crosslinks themselves.

Finally, the title does not fully reflect the conclusions of the work.

Reviewer #2

(Remarks to the Author)

The study by Zambri et al. reports on a novel role of MreB in growth in a normally polarly growing bacterium. It is normally thought that bacteria either grow from the poles in an MreB-dependent manner, or along the lateral wall using MreB. Here the authors show that the actinobacteria, *Streptomyces* can undergo two different forms of growth, one polar (vegetative) and one polar and lateral (exploratory). This may be the first time that both forms of growth have been shown to occur at the same time. In addition, the authors show that the thickness of the cell wall changes between these two growth patterns as well as the actual structure of the wall.

The paper is well organized and well written. However, there are some changes to the figures that I think would enhance the manuscript.

Fig. 1: Day 5 of exploratory growth has a very thin cell wall and day 11 has a thick cell wall. It would be nice to see the change in cell wall composition between these points rather than day 7. Also what do you think causes the change in cell wall thickness if not the structure of the wall? Is minimal crosslinking changes between day 7 and 11

Fig. 2: It would be helpful to see an early time point in C (maybe day 4) before lysis of the mreB mutant. At day 10 I do not see any fluorescent signal at all. Are you saying that the entire colony has lysed at this point? The text reads more like only section of the colony have lysed and therefore I would expect to see zones of fluorescence. Day 4 has equal intensity (2C graph) so it would be nice to see this in the pictures of the colonies to confirm that the mreB mutant does produce signal.

Have the authors given any thoughts to the stoichiometry of MreBCD being important. The fact that loss of mreB is different than the entire operon suggests that there maybe effects of expressing the downstream genes without MreB around. Perhaps lysis is dependent on MreB alone but colony size is due to having too much of one of the other proteins. Can the authors measure MreB levels (western would be best) in the complemented strain as the colony size is not complemented only the lysis/wrinkling?

Line 219-220: The authors speculate that the mreB mutant may lyse more easily. This seems easily testable and should be done.

FDAA and Halo tags come in a variety of colors. Can the authors colocalize MreB and the FDAA in the same cells?

Fig.4: It would be nice to see the cell wall patterning during veg growth of the mreB mutant (similar to fig 5). Presumably there would be no difference?

Reviewer #3

(Remarks to the Author)

In this work, Zambri et al. study cell wall synthesis during exploratory growth of *Streptomyces*. They demonstrate that during this growth phase, the thickness of the exploring hyphal cell wall increases, coinciding with changes in the composition of the cell wall crosslinks. Deletion of mreB1 results in defects in exploratory behavior, as well as the changes in the cell wall changes related to this mode of growth. Using fluorescent D-amino acids, they show that *Streptomyces* exhibit lateral cell wall synthesis during exploratory growth, in addition to the classical polar growth also observed during vegetative growth. Moreover, analysis of MreB1 localization reveals that it moves directionally, usually perpendicular to the long axis of the hypha, suggesting a role for MreB1 in lateral cell wall synthesis during exploratory growth. The authors also analyze the conservation of MreB in actinobacteria.

The manuscript is well-written and presents the data clearly. This work makes significant contributions to our understanding of cell wall synthesis in actinobacteria, particularly as *Streptomyces* were thought to undergo only polar growth. I believe this study has the potential to move the field forward, however, a few points need to be strengthened, and I have highlighted them below:

- The localization of MreB1-Halo shows a pattern similar to that observed with FDAA staining. The authors must co-localize MreB1 with active site of peptidoglycan synthesis in the hyphae (MreB1-Halo and FDAA staining).
- If exploratory growth involves a redistribution of cell wall synthesis, is the mreB1 operon upregulated in the hyphae undergoing this growth? If I am correct, previous research did not identify mreB1 as a gene upregulated during exploration. This is something that the authors can study using transcriptional fluorescent reporters.
- The analysis of the mreB cluster is intriguing and suggests lateral cell wall synthesis in non-sporulating actinobacteria, but it still feels speculative, and does not add much on its own. Including FDAA labeling of PG synthesis in representative species, without requiring genetic manipulation, would significantly enhance the impact of this research beyond *Streptomyces*.

Some minor comments:

- Why does the complemented mreB1 mutant not fully restore the wildtype morphology during exploration? This could be due to polar effects on rodA and pbp from the insertion of the resistance cassette upstream. Have the authors tried complementing the mutant with the entire operon?
- Is there a specific reason the authors did not use the commonly used FDAA HADA?
- In Fig. 5A, the top panels showing mreB1 localization in the spores were a bit confusing. Please consider clarifying this in the legend.
- Was sporulation observed in liquid YP medium?
- It will be good to show that the PG synthesis machinery (RodA and bPBP) co-localizes and shows the same movement that MreB1. Have the authors look at the localization of these components in classical growth vs exploratory growth?

Decision Letter:

11th September 2024

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Dr Elliot,

Thank you for your patience while your manuscript "Bacteria grow using both polar and dispersed cell wall synthesis" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end

of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, several of the referees raised questions over whether the phenotype identified could solely be attributed to MreB1 alone, and recommended several experiments to demonstrate whether additional proteins encoded by genes in the mre operon might also contribute. These include analysing the involvement of rodA and bPBP. There were also requests for further supporting experiments, including analysing colocalisation of MreB1 with FDAA labelling, to strengthen the conclusions made. Referee #1 raised concerns regarding the peptidoglycan analyses, while Referee #3 requested evidence of lateral FDAA labelling in at least one other actinobacteria, to strengthen the suggestion that lateral cell wall synthesis is conserved in different species. We feel that these are critical points which would need to be addressed for us to further consider a revised manuscript, alongside the remaining issues outlined in the referees' reports, which are clear and should be straightforward to address.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at <https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data>.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc...), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>

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- * Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

- * If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at <http://www.nature.com/nmicrobiol/info/final-submission>. Refer also to any guidelines provided in this letter.

- * Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading or sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please use the link below to submit a revised paper:

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months).

In the meantime we hope that you find our referees' comments helpful.

Yours sincerely,

Reviewer Expertise:

Referee #1: peptidoglycan synthesis

Referee #2: bacterial cell biology

Referee #3: bacterial cell biology, streptomyces

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

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Reviewer #2 (Remarks to the Author):

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Some minor comments:

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- Is there a specific reason the authors did not use the commonly used FDAA HADA?
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- Was sporulation observed in liquid YP medium?
- It will be good to show that the PG synthesis machinery (RodA and bPBP) co-localizes and shows the same movement that MreB1. Have the authors look at the localization of these components in classical growth vs exploratory growth?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The additional experiments on the impact of individual genes in the mre operon on exploration strongly support the contribution of MreB1 in this process. The peptidoglycan analyses are now clearly and thoroughly described, and all my concerns have been

fully addressed.

Reviewer #2

(Remarks to the Author)

The new version of the manuscript has addressed many of the issues previously raised. However, there are a couple of issues that still should be addressed.

Major issues

1) The polarity of the mreB deletion should be addressed by complementing mreB from a stronger or inducible promoter. If the lack of full complementation is due to expression levels then this should fix the problem

2) I do not understand what was done for the TDL-Halo co staining experiment. Why were both stains not added at the same time? Why was there so much time between stains that the filaments grew as much as they did?

What does this experiment actually show? The point was to show that areas of TDL staining correlate with areas of MreB. I do not see this in the figure. If this argument is being made how was this conclusion made?

3) If the mreB cells are more susceptible to lysing as stated on line 218 then I would expect they are more sensitive to envelope targeting antibiotics or osmotic shock. This could easily be tested

Minor Issues

I find it difficult to see lysis of the mreB deletion colonies. Can you please use an arrow to indicate what is lysis on the colony. It is hard to distinguish this from changes in wrinkle pattern

Reviewer #3

(Remarks to the Author)

The authors have thoroughly addressed all my previous comments and questions, and I believe the revised version has significantly improved the overall impact of the manuscript, especially by showing that the lateral cell wall synthesis seems to be present outside Streptomyces in those actinobacteria that also encode mreB. I have just one minor suggestion related to figure presentation that could help the clarity of the data. In Figure 6B, for consistency with the other microscopy figures, consider separating the DIC and fluorescence channels. This would also help interpret the data more easily. This is only a minor suggestion, and I am happy to recommend the manuscript for publication.

Decision Letter:

20th May 2025

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Dr Elliot,

Thank you for your patience while your manuscript "Streptomyces grow using both polar and dispersed cell wall synthesis" was under peer-review at Nature Microbiology. It has now been seen by the 3 original referees, whose expertise and comments you will find at the end of this email. You will see from their comments below that while they find your work of interest, and they are mostly satisfied that the revisions have addressed their concerns, there are a few points that still remain. We are very interested in the possibility of publishing your study in Nature Microbiology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

From the reports below you will see that Reviewers #1 and 3 find that with the revisions made, the data support the conclusions, with only a minor comment regarding the presentation of one figure to aid clarity (mentioned by Reviewer #3). Reviewer #2 has a few remaining points, regarding the evidence for increased lysis in the mreB1 mutant strain, including to better indicate in the existing images where lysis is shown, and to use antibiotics or osmotic shock to provide further support for this point. They also ask for more information on the methods for the MreB1/TDL colocalisation assay and explanation of the findings (following on from their earlier request to explore this). We think that these are points that should be feasible to address with text edits or perhaps a small amount of experimental work. Reviewer #2 also asks for experiments with overexpression of MreB1 to see if this can fully complement the mutant strain - we would overrule this request as other work rules out the possibility of a contribution from other genes in the operon.

We hope this should be straightforward. We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at <http://www.nature.com/nmicrobiol/info/final-submission/>

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Some reduction could be achieved by focusing any introductory material and moving it to the start of your opening 'bold' paragraph, whose function is to outline the background to your work, describe in a sentence your new observations, and explain your main conclusions. The discussion should also be limited. Methods should be described in a separate section following the discussion, we do not place a word limit on Methods.

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Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc...), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>

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Figure legends must provide a brief description of the figure and the symbols used, within 350 words, including definitions of any error bars employed in the figures.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines](https://www.nature.com/nature-research/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading or sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

EXTENDED DATA FIGURES

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We hope to receive your revised paper within three weeks. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

Yours sincerely,

Reviewer Expertise:

Referee #1: PGN crosslinking structural analysis

Referee #2: Cell biology/cell division

Referee #3: cell biology/cell division

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

The additional experiments on the impact of individual genes in the mre operon on exploration strongly support the contribution of MreB1 in this process. The peptidoglycan analyses are now clearly and thoroughly described, and all my concerns have been fully addressed.

Reviewer #2 (Remarks to the Author):

The new version of the manuscript has addressed many of the issues previously raised. However, there are a couple of issue that still should be addressed.

Major issues

1) The polarity of the mreB deletion should be addressed by complementing mreB from a stronger or inducible promoter. If the lack of full complementation is due to expression levels then this should fix the problem

2) I do not understand what was done for the TDL-Halo co staining experiment. Why were both stains not added at the same time? Why was there so much time between stains that the filaments grew as much as they did?

What does this experiment actually show? The point was to show that areas of TDL staining correlate with areas of MreB. I do

not see this in the figure. If this argument is being made how was this conclusion made?

3) If the mreB cells are more susceptible to lysing as stated on line 218 then I would expect they are more sensitive to envelope targeting antibiotics or osmotic shock. This could easily be tested

Minor Issues

I find it difficult to see lysis of the mreB deletion colonies. Can you please use an arrow to indicate what is lysis on the colony. It is hard to distinguish this from changes in wrinkle pattern

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed all my previous comments and questions, and I believe the revised version has significantly improved the overall impact of the manuscript, especially by showing that the lateral cell wall synthesis seems to be present outside Streptomyces in those actinobacteria that also encode mreB. I have just one minor suggestion related to figure presentation that could help the clarity of the data. In Figure 6B, for consistency with the other microscopy figures, consider separating the DIC and fluorescence channels. This would also help interpret the data more easily. This is only a minor suggestion, and I am happy to recommend the manuscript for publication.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Dear authors,

It's all good for me. While I was (re)reading the revised version, I noticed that I forgot one mistake: in Fig. 1b, the L,D-crosslinks must connect two purple circles.

All the best!

Decision Letter:

Our ref: NMICROBIOL-24082355B

10th June 2025

Dear Dr. Elliot,

Thank you for submitting your revised manuscript "Streptomyces grow using both polar and dispersed cell wall synthesis" (NMICROBIOL-24082355B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Sincerely,

Reviewer #1 (Remarks to the Author):

Dear authors,

It's all good for me. While I was (re)reading the revised version, I noticed that I forgot one mistake: in Fig. 1b, the L,D-crosslinks must connect two purple circles.

All the best!

Version 3:

Decision Letter:

3rd July 2025

Dear Marie,

I am delighted to accept your Article "Streptomyces use both polar and dispersed cell wall synthesis during exploratory growth" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

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RESPONSE TO REVIEWERS' COMMENTS

EDITOR'S COMMENTS:

Thank you for your patience while your manuscript "Bacteria grow using both polar and dispersed cell wall synthesis" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, several of the referees raised questions over whether the phenotype identified could solely be attributed to MreB1 alone, and recommended several experiments to demonstrate whether additional proteins encoded by genes in the *mre* operon might also contribute. These include analysing the involvement of *rodA* and *bPBP*. There were also requests for further supporting experiments, including analysing colocalisation of MreB1 with FDAA labelling, to strengthen the conclusions made. Referee #1 raised concerns regarding the peptidoglycan analyses, while Referee #3 requested evidence of lateral FDAA labelling in at least one other actinobacteria, to strengthen the suggestion that lateral cell wall synthesis is conserved in different species. We feel that these are critical points which would need to be addressed for us to further consider a revised manuscript, alongside the remaining issues outlined in the referees' reports, which are clear and should be straightforward to address.

Thank you very much for your positive and constructive assessment of our work, and for the insightful and helpful comments from the reviewers. We have worked to address all comments, as indicated below. With respect to the critical points raised above:

- We have probed the role of other genes from the *mre* operon in association with exploration. We have deleted each of these genes individually – including both *bPBP* and *rodA* – and found that loss of these genes had little effect on exploration, with there being no discernable difference from the wild type phenotype for any but the *bPBP* mutant, and even that had only a subtle phenotypic effect. We also complemented the *mre* operon with just *mreB1*, and found that it restored wild type growth, suggesting that of the gene products encoded by the *mre* operon, MreB1 is the most important for exploration. These data are now presented in Figure S3B.
- We conducted co-localization experiments for MreB1 and TDL (the FDAA that worked well for exploring cultures), and found that the two overlapped nicely. These data are now included in Figure 5C
- Regarding our peptidoglycan analyses, we have added in additional methodological information, have included representative chromatograms (Figure S1C and D), and have clarified/corrected our labelling in both Figure 1 and Tables S1 and S2.
- We conducted FDAA labelling experiments for two slow-growing MreB-encoding actinobacteria (*Eggerthella lenta* and *Collinsella aerofaciens*). We found that after a short labelling period (5 min), the label - and presumably cell wall synthesis - was not confined to

the poles of the cells, suggesting a possible role for MreB in cell wall synthesis in these systems. These data are now presented in Fig. 6.

RESPONSE TO REVIEWER #1:

COMMENT 1: “The peptidoglycan analyses should be better described to convincingly demonstrate the structural changes associated with exploration and the deletion of the mreB operon. In Figure 1, the representation of the L,D-crosslink is incorrect (D-Ala4 should be absent in the donor peptide stem). The peptidoglycan analyses are not sufficiently detailed to support the conclusions: (i) the methods section should include MS and MS/MS; (ii) the abbreviations for mucopeptides in the dataset are not fully explained. Does “G” stand for glycine, GlcNAc, or both? Which mucopeptides were identified as 3-3 or 4-3 crosslinks? How was glycine in the side chain distinguished from glycine at the C-terminus of peptide stems? I suggest including representative chromatograms to strengthen the conclusions.”

Thank you for catching the error in Fig. 1 – we have removed the D-Ala4 from the donor peptide stem.

With respect to the analyses:

- i) We have added additional information into the methods section, including details on MS and MS/MS (lines 568-587).
- ii) We have changed our mucopeptide labelling to the Glauner’s nomenclature and have now defined all abbreviations in Tables S1 and S2. By adopting Glauner’s nomenclature we hope that the identification of 3-3 versus 4-3 crosslinks is now more accessible. In *Streptomyces*, the basic peptidoglycan (PG) building block typically consists of GlcNAc-MurNAc-L-Ala-D-Glu-DAP(Gly)-D-Ala-D-Ala (Hugonnet et al., 2014, <https://doi.org/10.1128/aac.02329-13>; Schleifer & Kandler, 1972, doi: 10.1128/br.36.4.407-477.1972), which includes a glycine residue attached to the meso-diaminopimelic acid (DAP) in the side chain [DAP(Gly)]. Further modifications to PG were identified by comparing the masses of the mucopeptides with those of the basic building block and the previously published comprehensive analysis of *S. coelicolor* peptidoglycan structure (doi: 10.1128/JB.00290-18). We have now included representative chromatograms (with peaks numbered and corresponding to Table S2) in Figure S1.

COMMENT 2: The deletion of mreB1 leads to a growth defect on YP medium. The authors attribute this defect solely to mreB1 deletion, but complementation does not seem to fully restore the wild-type phenotype. The text states: “Introducing a wild-type copy of mreB1 at a heterologous site in the chromosome restored a more wild-type-like wrinkling pattern and alleviated the apparent cell lysis, although colonies remained smaller than wild-type.” As shown, the phenotype of the strain lacking the entire mre operon does not seem to differ from that of the Δ mreB1 strain. Therefore, the mreB1 mutant phenotype might be due to a polar effect on downstream genes in the mreB operon. A markerless in-frame deletion of mreB1

(similar to what was done for the *mreB2* mutant) would greatly support the conclusions. In Figure 2, the $\Delta mreB1::mreB1$ strain producing mNeonGreen is missing, which raises further doubts about the specific contribution of *mreB1*. This control should be included. Exploration growth is convincingly affected by the deletion of the *mre* operon, but attributing the phenotype to a specific gene remains unsupported.

Based on the data provided in Figures S3A, S4 and S6C, our interpretation is that the phenotypes of the *mreB1* mutant and the *mre* operon mutant do differ. To more clearly demonstrate the relative importance of MreB1 compared with the other proteins encoded within the *mre* operon (and to address the possible contribution of polar effects), we generated additional individual deletion strains for *mreC*, *mreD*, *bPBP* and *rodA* (each gene in the operon) and assessed their exploring phenotypes (Fig. S3B). The only strain that presented a non-wild type-like phenotype (other than $\Delta mreB1$ and the Δmre operon) was the $\Delta bPBP$ strain, which had a subtle phenotype with slightly increased surface area occupancy and altered wrinkling pattern. This differed from the reduced exploration phenotype observed for the *mreB1* mutant. We acknowledge that we were unable to fully restore wild type exploration upon complementation of the *mreB1* mutant with wild type *mreB1* expressed from its native promoter at a heterologous locus; however, in experiments described below, we now know that MreB1 levels in the complemented strain are much lower than when MreB1 is expressed from its native locus, which could explain the partial complementation observed.

We have also modified Figure 2 and now include a complemented *mreB1* mutant strain that expresses mNeonGreen. The revised figure shows both plate images and quantified fluorescence.

COMMENT 3: The increase of D,D-crosslinks in the exploration mode and the loss of TDL labeling in the *mreB* mutant suggest an important role for the bPBP encoded by the *mreB* operon in this mode of growth. If this is the case, fast-growing hyphae would require a fast-acting enzyme (the bPBP) to sustain rapid growth, rather than a slower transpeptidase (an L,D-transpeptidase). This phenomenon would be distinct from the peripheral growth of *Mycobacteria*, which relies on the maturation of PBP-mediated peptidoglycan polymerization by L,D-transpeptidases (Elife, 2018, Oct 16:7.doi: 10.7554/eLife.37516). Regarding Figure 4, what is the labeling profile of an 11-day-old culture? If labeling occurs, this could suggest that other PBPs partially compensate for the loss of function of the bPBP.

We agree that the bPBP encoded in the *mre* operon may contribute to exploration, given the subtly altered exploration phenotype associated with our single $\Delta bPBP$ strain. However, given that we also found that complementation of the *mre* operon mutant with just *mreB* was sufficient to restore wild type-like exploration, this suggests that the bPBP encoded in the *mre* operon is not critical for MreB activity. Given the overall decrease in L,D-crosslinks during exploration (for all strains), we do not think that L,D-transpeptidases are responsible for driving exploratory growth. We have repeatedly attempted TDL labelling with 11-day old exploring cultures; however, we have struggled to observe much cell labelling, making it challenging to draw meaningful conclusions. We attribute this to the age of the colonies and a significantly reduced proportion of cells that are actively growing (a prerequisite for FDAA labelling). As shown in Fig. S5B, over 50% of the cell population did not appear to be actively growing at 5 days, based on the lack of TDL labelling; we anticipate this number to be even higher at 11 days.

COMMENT 4: In the introduction: “DivIVA is broadly conserved among Gram-positive bacteria (Hammond, White & Eswara, 2019), but its role in cell wall synthesis is confined to the actinobacteria, where it is an essential protein (Flärdh, 2003).” This sentence is confusing, as DivIVA is also involved in cell wall synthesis in other bacteria (e.g., streptococci).

We have adjusted the language to highlight the importance of DivIVA in the actinobacteria without disregarding its biological contributions in other phyla. We now state in lines 36-38: *DivIVA is broadly conserved among Gram-positive bacteria (Hammond, White & Eswara, 2019), but in the actinobacteria, unlike in other systems, its role in cell wall synthesis is essential for viability (Flärdh, 2003).*

COMMENT 5: “These glycan strands are joined via their NAM-affixed peptide stems, with these stems in turn being linked together by either D,D-crosslinks catalyzed by penicillin-binding proteins (PBPs) or L,D-crosslinks catalyzed by L,D-transpeptidases (Glauner, Höltje & Schwarz, 1988).” The formation of crosslinks is catalyzed, rather than the crosslinks themselves.

We have corrected this sentence (lines 86-88).

COMMENT 6: Finally, the title does not fully reflect the conclusions of the work.

We have modified the title of our manuscript in a way that we hope accurately reflects our findings and conclusions.

REVIEWER #2 (REMARKS TO THE AUTHOR):

The study by Zambri et al. reports on a novel role of MreB in growth in a normally polarly growing bacterium. It is normally thought that bacteria either grow from the poles in an MreB-dependent manner, or along the lateral wall using MreB. Here the authors show that the actinobacteria, *Streptomyces* can undergo two different forms of growth, one polar (vegetative) and one polar and lateral (exploratory). This may be the first time that both forms of growth have been shown to occur at the same time. In addition, the authors show that the thickness of the cell wall changes between these two growth patterns as well as the actual structure of the wall.

The paper is well organized and well written. However, there are some changes to the figures that I think would enhance the manuscript.

COMMENT 1: Fig. 1: Day 5 of exploratory growth has a very thin cell wall and day 11 has a thick cell wall. It would be nice to see the change in cell wall composition between these points rather than day 7. Also what do you think causes the change in cell wall thickness if not the structure of the wall? Is minimal crosslinking changes between day 7 and 11.

We agree that comprehensive peptidoglycan compositional analysis over an exploration time course would almost certainly provide valuable information. We would note, however, that there

are significant differences in wall width at day 7 versus day 11 during growth on YP medium. We also conducted cell wall analyses of exploring cultures grown with yeast on YPD medium (YPDy), where exploration takes longer to initiate. Early exploration on YP (e.g., day 5 and earlier) would be equivalent to day 11 on YPDy – the data for which are presented in Figure S1A. There are significant changes in crosslinking when comparing day 11 and 16 of YPDy exploring cultures, suggesting that the structure of the wall is indeed changing as exploration proceeds. We have worked to ensure this information is more clearly communicated within our results (lines 101-104).

COMMENT 2: Fig. 2: It would be helpful to see an early time point in C (maybe day 4) before lysis of the mreB mutant. At day 10 I do not see any fluorescent signal at all. Are you saying that the entire colony has lysed at this point? The text reads more like only section of the colony have lysed and therefore I would expect to see zones of fluorescence. Day 4 has equal intensity (2C graph) so it would be nice to see this in the pictures of the colonies to confirm that the mreB mutant does produce signal.

For Figure 2C, we now include a time-course, showing plate images for days 4 and 10, and a graph in which colony fluorescence is quantified at days 2, 4, 10 and 12. As is apparent from the figure, there are still fluorescent regions within the colony, suggesting that wholesale colony lysis is not happening.

COMMENT 3: Have the authors given any thoughts to the stoichiometry of MreBCD being important. The fact that loss of mreB is different than the entire operon suggests that there maybe effects of expressing the downstream genes without MreB around. Perhaps lysis is dependent on MreB alone but colony size is due to having too much of one of the other proteins.

We agree that the stoichiometry of the system may well be important, and we have expanded on this possibility in our discussion (lines 445-451)

Can the authors measure MreB levels (western would be best) in the complemented strain as the colony size is not complemented only the lysis/wrinkling?

To compare MreB levels, we used our MreB1-Halo(SW) strain, where the associated gene was integrated in place of the wild type gene at its native locus. We also amplified this fusion, together with the native *mreB1* promoter, and introduced it into an integrating plasmid (the same vector used for the *mreB1* complementation experiments). We performed western blots for MreB, using Halo-specific antibodies. As a negative control for antibody specificity, we used a wild type strain expressing untagged MreB. We found that MreB-Halo(SW) was significantly less abundant when expressed at the ectopic locus than from its native chromosomal location. This could explain why the *mreB* complementation construct only partially rescued the mutant phenotype. We now include these data in Figure S3, and also present them below.

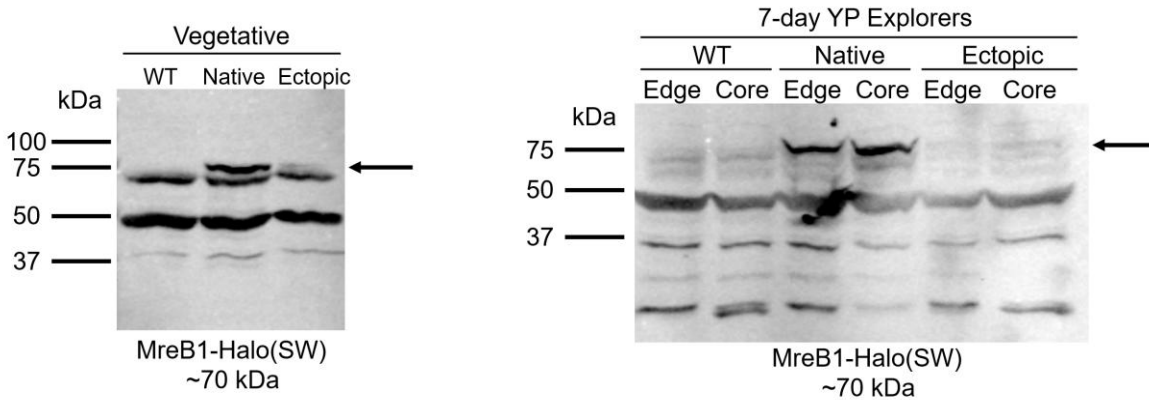


Fig S3A: Western blot using α -HaloTag antibodies (1:5000) comparing MreB-Halo(SW) (~70 kDa) levels between strains expressing the construct at its native locus (Native) and at an ectopic locus (Ectopic). The wild type (WT; non-tagged strain) served as a negative control for antibody specificity. The black arrow indicates the MreB-Halo(SW)-specific band. Both vegetative (classic growth conditions) and exploring conditions were tested. Chemiluminescent HRP-conjugated secondary antibodies were used for detection.

COMMENT 5: Line 219-220: The authors speculate that the *mreB* mutant may lyse more easily. This seems easily testable and should be done.

The loss of cellular fluorescence in the *mreB1* mutant strain (Figure 2C), and the increase in lysed filaments observed in our scanning electron microscopy images, supported a role for cell lysis. To further bolster this claim, we have added a representative transmission electron microscopy image for wild type and our *mreB1* mutant (Fig. S3D) at the 11-day timepoint. These images reveal significant differences between the strains, where the *mreB1* mutant is characterized by abnormal cell shapes and considerable debris, which is presumed to be the result of cell lysis.

COMMENT 6: FDAA and Halo tags come in a variety of colors. Can the authors colocalize MreB and the FDAA in the same cells?

This is not a straight-forward experiment given the differing technical requirements for MreB and FDAA imaging. With FDAA-labelling, this typically involves fixing the cells after labelling to preserve the incorporation pattern at the time of labelling. Cell fixation, however, leads to both loss of MreB1 movement and dissociation of MreB filaments.

To circumvent these challenges, we attempted to co-localize MreB1 movement with FDAA incorporation in the absence of fixation. We used TDL (TRITC channel) and purchased a HaloTag ligand in the Far-Red channel (JF646, Cy5 channel). We observed excellent overlap between MreB1 and the FDAA labelling pattern. In Video S11, we can see abundant MreB1 directional movement in cells that have an FDAA side wall labelling pattern. We note that in this video file, the localization of MreB1 extends beyond the boundary of FDAA labelling. Consequently, the cell lengths appear to be different in the TDL images than they do in the MreB1-Halo(SW) channels. This is because unincorporated FDAA's must be washed away for proper visualization, and because we were unable to fix the cells (in order to get meaningful MreB1 localization), the cells continued to grow after the FDAAs had been washed away and before we could begin hyphal visualization. This new

growth would not be labelled with FDAAs, but could contain active MreB1. We now include these data in Figure 5C and Video S11.

COMMENT 7: Fig.4: It would be nice to see the cell wall patterning during veg growth of the mreB mutant (similar to fig 5). Presumably there would be no difference?

We have now added in representative images of TDL-labelled WT and $\Delta mreB1$ strains grown in vegetative conditions (Figure S5D). We observed no major differences in labelling patterns for each.

REVIEWER #3 (REMARKS TO THE AUTHOR):

In this work, Zambri et al. study cell wall synthesis during exploratory growth of *Streptomyces*. They demonstrate that during this growth phase, the thickness of the exploring hyphal cell wall increases, coinciding with changes in the composition of the cell wall crosslinks. Deletion of *mreB1* results in defects in exploratory behavior, as well as the changes in the cell wall changes related to this mode of growth. Using fluorescent D-amino acids, they show that *Streptomyces* exhibit lateral cell wall synthesis during exploratory growth, in addition to the classical polar growth also observed during vegetative growth. Moreover, analysis of MreB1 localization reveals that it moves directionally, usually perpendicular to the long axis of the hypha, suggesting a role for MreB1 in lateral cell wall synthesis during exploratory growth. The authors also analyze the conservation of MreB in actinobacteria.

The manuscript is well-written and presents the data clearly. This work makes significant contributions to our understanding of cell wall synthesis in actinobacteria, particularly as *Streptomyces* were thought to undergo only polar growth. I believe this study has the potential to move the field forward, however, a few points need to be strengthened, and I have highlighted them below:

COMMENT 1: The localization of MreB1-Halo shows a pattern similar to that observed with FdAA staining. The authors must co-localize MreB1 with active site of peptidoglycan synthesis in the hyphae (MreB1-Halo and FdAA staining).

Please see our response to Reviewer 2, comment 6.

COMMENT 2: If exploratory growth involves a redistribution of cell wall synthesis, is the mreB1 operon upregulated in the hyphae undergoing this growth? If I am correct, previous research did not identify mreB1 as a gene upregulated during exploration. This is something that the authors can study using transcriptional fluorescent reporters.

Previous reports on gene expression levels during exploration have not identified *mreB1* as a gene that is strongly upregulated during exploration, compared with vegetative conditions. We also find this to be an interesting point, but do not yet have answers. In our western blots (above and in

Figure S3A), we find that during exploration – and during classic vegetative growth - MreB1 is readily detectable, and we see active movement when visualizing our MreB1-Halo(SW) fusion. While much of our data point to MreB1 being less involved/important in classical vegetative development, we are not claiming that it is not active at this stage, and future work will no doubt help to more fully elucidate whether there are roles for MreB1 beyond sporulation and exploration in *Streptomyces* species.

COMMENT 3: The analysis of the mreB cluster is intriguing and suggests lateral cell wall synthesis in non-sporulating actinobacteria, but it still feels speculative, and does not add much on its own. Including FDAA labeling of PG synthesis in representative species, without requiring genetic manipulation, would significantly enhance the impact of this research beyond *Streptomyces*.

We performed FDAA labelling with two species of Actinobacteria that encode MreB: *Collinsella aerofaciens* and *Eggerthella lenta*. We found both organisms showed an incorporation pattern that extended beyond the cell pole (Fig. 6B). While we are not able to conclude definitively that this expanded labelling pattern is due to the activity of MreB, it does support the proposal that actinobacterial growth is not confined to the cell poles.

COMMENT 4: Why does the complemented mreB1 mutant not fully restore the wildtype morphology during exploration? This could be due to polar effects on rodA and pbp from the insertion of the resistance cassette upstream. Have the authors tried complementing the mutant with the entire operon?

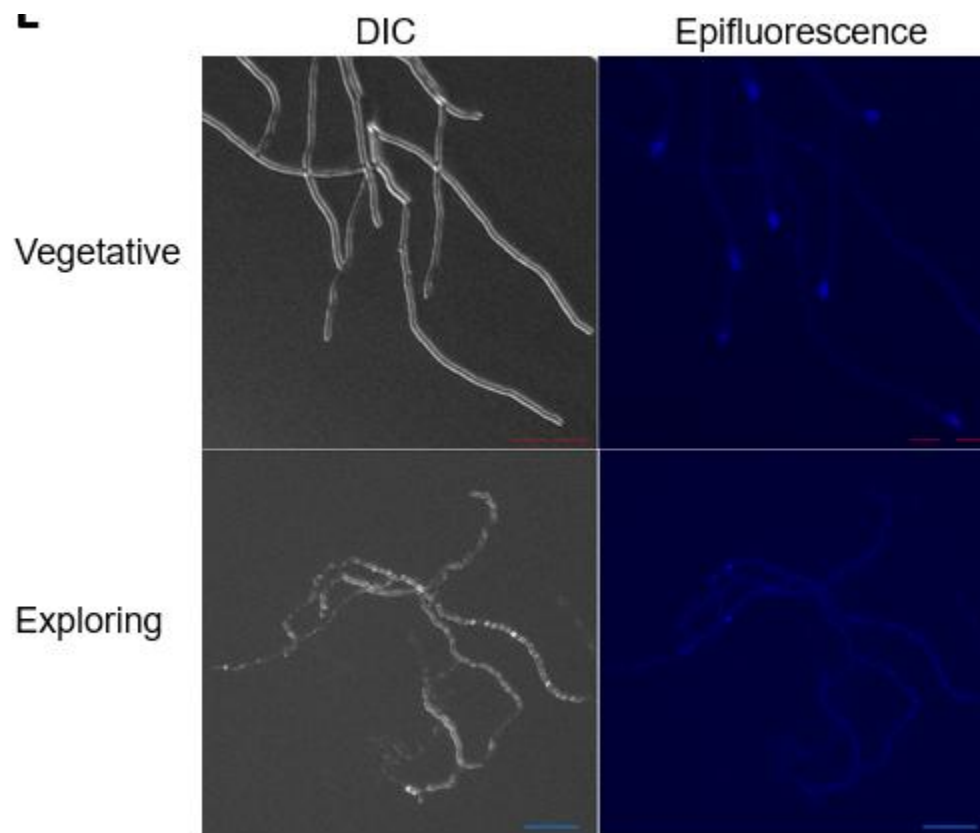
Please see our responses to Reviewer 1, Comment 2, and Reviewer 2, Comment 3. In lieu of complementing the *mreB1* mutant with the full operon (as we established that the effects stemming from the loss of *mreB1* were more profound than the mutation of all other genes in the operon), we tested whether the *mre* operon mutant could be complemented with *mreB1*. We found that complementation was nicely restored in this strain. We are unsure about why we did not see equivalent complementation success for our *mreB1* mutant alone, but propose that MreB1 may be expressed to higher levels in this background. We now address this in our discussion (lines 445-451).



Representative images of wild type + empty vector (WT + EV), Δmre operon strain + empty vector (Δmre + EV) and the Δmre operon strain complemented with *mreB1* under the control of its native promoter, showing a lack of cell lysis and a more wild type-like phenotype.

COMMENT 5: Is there a specific reason the authors did not use the commonly used FDAA HADA?

We attempted HADA labelling on multiple occasions. While we were able to successfully label classically growing vegetative hyphae of *S. venezuelae* with HADA, we were never able to detect consistent labelling of exploring cultures – either those undergoing conventional exploration on solid medium, or those grown in exploration-proxy liquid YP medium. We also found TDL to be far more photostable than HADA, making the imaging of TDL-labelled cells much easier. Please see below for a comparison of vegetative and exploring *S. venezuelae* labelled with HADA. The vegetative hyphae show nice polar labelling, while we only occasionally observed small randomly placed puncta with very little overall incorporation in exploring hyphae.



COMMENT 6: In Fig. 5A, the top panels showing *mreB1* localization in the spores were a bit confusing. Please consider clarifying this in the legend.

We have added an additional label in the inlayed image in Fig. 5A. We hope this improves clarity.

COMMENT 7: Was sporulation observed in liquid YP medium?

Sporulation was observed in liquid YP, but not to the extent that was observed in liquid MYM. Select hyphae begin to sporulate, but we do not observe the culture-wide transition into sporulation that is seen in liquid MYM.

COMMENT 8: It will be good to show that the PG synthesis machinery (RodA and bPBP) co-localizes and shows the same movement that MreB1. Have the authors look at the localization of these components in classical growth vs exploratory growth?

We were unable to identify a mutant phenotype for RodA, and consequently we would be unable to validate functionality of any fusion protein used to follow localization. Consequently, we focussed our attention on the bPBP, given its subtle defect in exploration compared with wild type. We created an mScarlet-bPBP fusion, and we introduced this construct either into its native locus (replacing the wild type copy), or into the *bPBP* mutant at a heterologous site, under the control of a constitutively expressed promoter. Unfortunately, we were unable to detect fluorescence associated with strains expressing either of these constructs. Given the subtle phenotype associated with the bPBP mutation, we were also not able to confidently determine whether these fusions were functional, or if the issue was with the stability of the fusion construct (the fluorescent tag might be being cleaved?) or with the expression of this gene. Considering that the *mre* operon mutant (lacking both RodA and bPBP) could be complemented with *mreB1* alone, this also suggested that MreB1 must be able to act with other biosynthetic enzymes. Determining what proteins associate with MreB (and with DivIVA) is a major priority for future work, as little is known about the enzymatic components making up either the MreB-guided 'elongasome' or the DivIVA-mediated polarisome.

Response to Reviewers' Comments

Editor's comments:

From the reports below you will see that Reviewers #1 and 3 find that with the revisions made, the data support the conclusions, with only a minor comment regarding the presentation of one figure to aid clarity (mentioned by Reviewer #3). Reviewer #2 has a few remaining points, regarding the evidence for increased lysis in the *mreB1* mutant strain, including to better indicate in the existing images where lysis is shown, and to use antibiotics or osmotic shock to provide further support for this point. They also ask for more information on the methods for the MreB1/TDL colocalisation assay and explanation of the findings (following on from their earlier request to explore this). We think that these are points that should be feasible to address with text edits or perhaps a small amount of experimental work. Reviewer #2 also asks for experiments with overexpression of MreB1 to see if this can fully complement the mutant strain - we would overrule this request as other work rules out the possibility of a contribution from other genes in the operon.

We have made the change requested by Reviewer #3, and have provided the additional experiments/information requested by Reviewer #2. We are grateful for your assessment that the additional experiment with overexpressing MreB was not necessary.

Reviewer #1 (Remarks to the Author):

The additional experiments on the impact of individual genes in the *mre* operon on exploration strongly support the contribution of MreB1 in this process. The peptidoglycan analyses are now clearly and thoroughly described, and all my concerns have been fully addressed.

Thank you!

Reviewer #2 (Remarks to the Author):

The new version of the manuscript has addressed many of the issues previously raised. However, there are a couple of issues that still should be addressed.

Major issues:

1) The polarity of the *mreB* deletion should be addressed by complementing *mreB* from a stronger or inducible promoter. If the lack of full complementation is due to expression levels then this should fix the problem.

We agree that this experiment would definitively show that the observed phenotype was due to the *mreB1* mutation; however, we would note that (i) the original complementation construct was sufficient to suppress colony lysis; (ii) deletion of all other genes (individually) from the *mre* operon had at most a subtle effect on exploration, and (iii) complementation of the *mre* operon with the *mreB1* gene alone could restore a wild type phenotype. Collectively, these results suggest that the exploration defect associated with the *mreB1* gene is largely due to the loss of *mreB1*.

2) I do not understand what was done for the TDL-Halo co staining experiment. Why were both

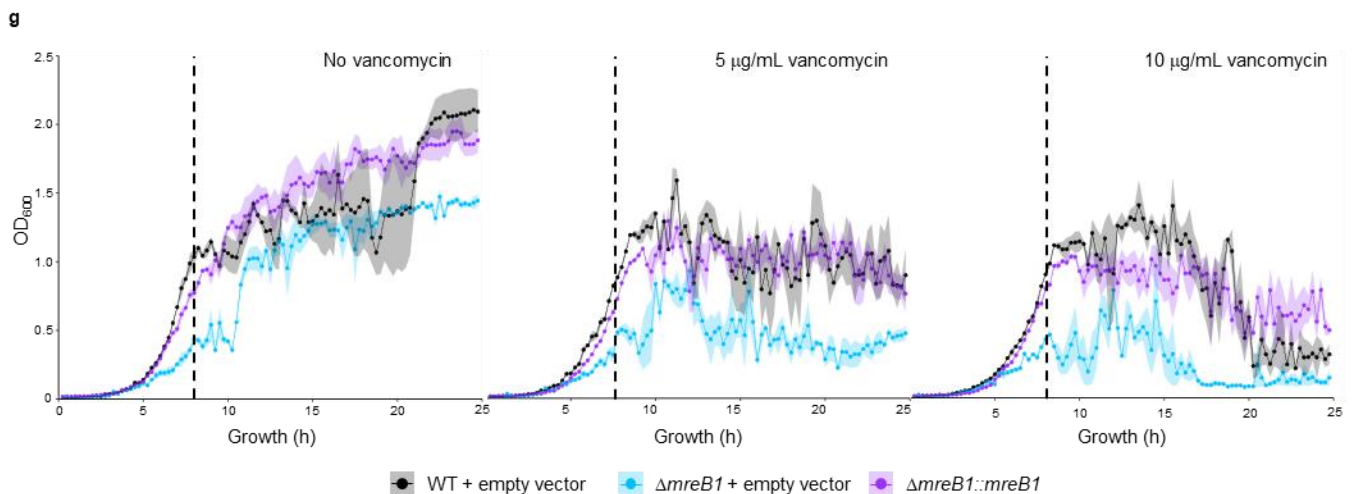
stains not added at the same time? Why was there so much time between stains that the filaments grew as much as they did?

What does this experiment actually show? The point was to show that areas of TDL staining correlate with areas of MreB. I do not see this in the figure. If this argument is being made how was this conclusion made?

We did not add both stains at the same time, as they require different incubation periods – the Halo ligand required a 30 min incubation, while TDL required a 5 min incubation – although we timed these so both incubations finished at the same time. The discrepancy in the label localization came from the fact that the TDL stain requires significant washing, and we were unable to fix the cells after labelling (MreB filament integrity is compromised when cells are fixed). Consequently the cells kept growing in the absence of label during the washing process. For all other experiments using the TDLs, we fixed the cells prior to washing, so were able to capture labelling at that precise moment in time. What the experiment is showing is that in the hyphae exhibiting significant growth (gap between TDL label and cell end), there is also abundant MreB1 labelling – including in the region that had continued growing in the absence of the label, suggesting that this growth has been driven (at least in part) by MreB1. We have included additional information in the Methods section to help in clarifying what we did and why (lines 426-429 in the final revised manuscript or lines 638-644 in the marked-up manuscript).

3) If the *mreB* cells are more susceptible to lysing as stated on line 218 then I would expect they are more sensitive to envelope targeting antibiotics or osmotic shock. This could easily be tested.

We have now compared the susceptibility of wild type, *mreB1* mutant, and *mreB1* complemented strains, to the cell wall-targeting antibiotic vancomycin. We found that, as expected, the *mreB1* mutant was more susceptible to vancomycin than either the wild type or *mreB1* complemented strain. These data are now presented in Figure S6C, and are shown below:



Minor Issues:

I find it difficult to see lysis of the *mreB* deletion colonies. Can you please use an arrow to indicate what is lysis on the colony. It is hard to distinguish this from changes in wrinkle pattern.

The lysis is easier to see in the movies than it is in the still image. To help emphasize the lysed regions around the colony periphery, we have now outlined the colonies in **Figure 2C** with a blue dotted line, to help in better detecting the lysed regions (which are no longer fluorescent in the *mreB1* mutant).

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed all my previous comments and questions, and I believe the revised version has significantly improved the overall impact of the manuscript, especially by showing that the lateral cell wall synthesis seems to be present outside *Streptomyces* in those actinobacteria that also encode *mreB*. I have just one minor suggestion related to figure presentation that could help the clarity of the data. In Figure 6B, for consistency with the other microscopy figures, consider separating the DIC and fluorescence channels. This would also help interpret the data more easily. This is only a minor suggestion, and I am happy to recommend the manuscript for publication.

We have now separated out the DIC and fluorescence channels in Figure 6B, as requested.