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Bacteria elicit a phage tolerance response subsequent to infection of their neighbors

Sigal Ben-Yehuda, Elhanan Tzipilevich, Osher Pollak-Fiyaksel, and Bushra Shraiteh
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **19th Aug 2021**

Dear Prof. Ben-Yehuda,

Thank you again for the submission of your manuscript entitled "Bacteria elicit a phage tolerance response subsequent to infection of their neighbors" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, all three referees are very positive towards your work but point out to some concerns, mostly minor, that will require your attention before your manuscript can be published in The EMBO Journal. Based on the overall interest expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

David

David del Alamo, PhD.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is

needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

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

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

Additional information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 17th Nov 2021:

Link Not Available

Referee #1:

In the manuscript "Bacteria elicit a phage tolerance response subsequent to infection of their neighbors" Tzipilevich and Pollak-Fiyaksel et al. investigate the phenomenon of "shrinking plaques" over the course of phage infection in solid medium. The authors demonstrate that Bacillus subtilis displays transient resistance to infection by the phages SSP1 and Phi29. Importantly, while highly divergent, both phages utilize wall teichoic acid (WTA) as receptors during infection. The authors define the transient resistance as "tolerance" and quantify this trait as the ability of the bacteria to invade into the zone of clearance created by the phage at approximately 8 hrs post infection, resulting in plaque size reduction. The authors go on to demonstrate that tolerance is mediated by the sigma factor SigX, which, upon phage infection, upregulates the dlt operon thereby resulting in

modification of WTA and reduced phage binding/infection. Furthermore, when the authors engineer the bacteria to overexpress SigX prior to infection there is dramatic reduction in plaque formation, indicative of limited phage infection. The authors used a combination of genetics and microscopy to clearly demonstrate this phenomenon. This work is of good quality and the conclusions are mostly supported by the data. Below are a few points to help solidify conclusions reached.

Major Comments

1. One of the principal findings from this manuscript is that a secreted factor from phage-infected cells activates SigX-dependent gene expression. These conclusions are reached from data presented in Fig. 3 and S5, however, those experiments lack an important control. In addition to uninfected PY79 + BS12, the GFP signal from BS12 should be analyzed when BS12 is co-cultured with ΔyueB +/- phage infection. If infected bacteria produce a diffusible signal that activates SigX, then addition of phage to a ΔyueB + BS12 co-culture should have no effect of BS12 GFP production. This experiment rules out a competing hypothesis that a component of the phage lysate used for infection is responsible for activating SigX.
2. The fluorescent images of full plaques, such as those presented in Fig. 1E, are very impressive and a large part of the results put forward by the authors. However, these images could be greatly improved by expanding the field of view (data that may already be available). At present, it appears the entire plate overlay becomes dominated by WT at 16 hours post infection. However, based on the authors conclusions, only the phage-adjacent bacteria invading the plaque should be WT and the surrounding bacteria should remain an equal mixture of WT and SigX. Can this be shown to justify the authors conclusions? If not, an alternative explanation for the observations in this manuscript could be that ΔsigX displays a growth disadvantage only when co-cultured with WT. At present, the growth rates of WT and ΔsigX are investigated in monoculture, but the phage infections are in competition. A more appropriate comparison would be demonstrating there is no competitive defect for ΔsigX in co-culture.
3. Can the authors better explain the SPP-lysin-yfp strain and results? This reviewer's understanding of lysin proteins is that they are secreted and do not mark the phage virion but rather the remnants of phage infection. If that is the case, could data in Fig. S1 and Movie S3 suggest that the rim of the plaque is an area of active replication instead of fully formed phage virions unable to infect sigX expressing cells? It is conspicuous that the YFP signal in Fig. S1 does not appear to constrict yet is empty in the center of the plaque. These possibilities could be accounted for by altering the language in Page 5 Line 22 from "signifying that bacteria at the rim could withstand the presence of phages" to something that accounts for the decreased phage infection kinetics observed later in the manuscript.

Minor Comments

1. In general, there is a lack of expected details and genetic controls presented. For example, many mutations appear to not be complemented in this study. Are fluorescent protein fusions of SigX functional? The authors should try to add these and other details.
2. The x-axis of many graphs and the timepoints of images shown are inconsistent throughout the manuscript and make comparisons difficult. For example, Fig. 2D, 4A, 5A (I think this should be min not hr?), S5C, and S7A all plot similar experiments but often have different x-axis ranges. Can these and other graphs be standardized?
3. Fig. 3A x-axis spelling error
4. This reviewer found the transition to why extracellular sigma factors were investigated difficult to follow. Further, I was disappointed in the lack of discussion for other SigX-regulated genes, such as ywbO. While not critical, if there is sufficient space, I would appreciate some discussion here.
5. On page 7 line 9 the word "infection" and should say "infected"
6. For Figure S8B the labels on the two images are the same. This reviewer believes the images are meant to represent two different time points and thus the labels should be altered.

Non-essential Suggestions

1. One additional note, but by no means a necessary requirement, have the authors considered treating B. subtilis with sub-lethal concentrations of antibiotics/antimicrobial peptides to induce SigX expression? Do these stimuli alter phage tolerance? This might be an exciting extension of the data presented. But again, this is outside of the scope of the current study and perhaps work that is already in progress.

Referee #2:

The work developed by Tzupilevich and colleagues is exciting and well designed. They bring techniques that will fuel novel discoveries within the group and others who investigate in this space. This manuscript explores bacteria's first line of defense against phages, which the authors point out as mostly unexplored and uncharacterized. With some of the community focusing on simply cataloguing new defense systems, studies like these are a breath of fresh air, getting us closer to the true ecological understanding of phage-host interactions. I would therefore like to congratulate the authors for this work. It was a pleasure to read, and therefore it is my pleasure to recommend this paper for publication after the authors address some minor

recommendations. I understand that some of the suggestions are at the rim of this manuscript, some intended to stimulate discussion, others to improve the clarity to the reader.

Recommendations:

1) The work focuses on studying phage-host interaction on solid media. When comparing the agarose pad results with the plate data it is unclear if the experiments are done in solid media or semi-solid (low agar percentage in double-layer agar technique). I recommend the authors to make this clearer in Material and Methods, and change the text if these are comparing semi-solid conditions with solid media;
2) What is the number of phages in the center vs the rim of infection?
3) The authors report that the isolated bacteria from the rim are phage sensitive but data is not shown. I suggest adding this data to the manuscript;
4) Could the authors make it clearer in line 7-8 of page 6 that the hypothesis is linked to cell envelope stress?
5) Do the authors know if phage replication is not affected at 48C? In Phi29, the burst size at 45C in a different strain has been shown to reduce from 570 to 260. What about in this strain for both phages?
6) I suggest that the authors revisit the word transcription on line 13 page 8. Perhaps the word 'expression' would be better suited to the observation of fluorescent signal measurements that are knowingly affected by protein turnover rates.

Suggestions:

1) The two phages used in this study have different reported burst sizes: 200-250 and 570, SPP1 and Phi29, respectively. Can the authors comment on the potential effect on the constrain reported and phage spreading?
2) The authors suggest a new term: phage tolerance response. Considering that all isolated bacteria from the rim are phage sensitive, do the authors consider that the mechanism of sensing and spreading initiates a cascade of process leading to non-reversible mutations in the community, or that is more likely that the phage pressure at the rim will select for these mutants?;
3) The schematic Figure 3A and 3B should be improved to help the reader understand better the plotted results;
4) For clarity I would suggest that the authors would represent the individual measurements in Figure 4, 5, S5 and S6;
5) Figure S7 does not seem color blind friendly and would be better if the authors would plot the average.

Typo:

Please correct the word infection in Figure 3A.

Referee #3:

Tzipilevich et al. used fluorescent microscopy to monitor phage plaque dynamics over time in B. subtilis and two of its phages, SPP1 and phi29. They find that after plaques expand, they restrict in diameter, indicating bacterial growth back into the cleared zone. The re-taking of the plaque by bacteria was linked to the activity of the sigma factor sigX, which controls the dlt operon that cell wall teichoic acids (TA). A soluble but undefined molecule able to diffuse through a membrane induced sigX-dependent TA cell wall remodeling.

The authors mention that plaque formation dynamics are understudied, and it is true. I have considered it myself many times: why don't plaques continue to grow and over take the whole plate? This was an interesting study and I believe it important to the field of phage biology in general. I have only minor comments:

In general, red and green images were difficult to differentiate (I am color blind).

Line 14, page 2: "propelled by transcriptional response" Are the authors referring to a phenotype here as opposed to a genotype?

Line 16-17, page 5: "...indicating that such a kinetic pattern is widespread." I suggest using a less definitive word (suggests instead of indicates) as only 2 phage species were tested, so it is difficult to claim something is very widespread.

Line 24, page 5 through line 1 page 6: "all tested bacteria remain phage sensitive." Please refer the reader to the data.

Fig 2A: more intuitive for readers (especially color-blind readers) if strain names were placed below the data rather than listed as a column to the side.

Line 14 page 6: Indicate to reader what conditions cells were grown when comparing wt and ΔsigX strains.

Line 17 page 6: "No difference in lysis kinetics was monitored when bacteria were infected.' "Observed" preferred over

"monitored"

Line 9 page 7: Authors state there is no difference in growth at 48C. Can the authors please clarify that they are comparing wt and sigX strains at 48C as opposed to comparing growth at two different temperatures?

Line 8 page 9: More details on reversible vs irreversible phage attachment would be welcome.

Line 7-8, page 13: I am not sure I fully agree with the statement "it is not entirely understood how activation of the CRISPR-Cas system is prompted." CRISPR-Cas is a general term that encompasses a lot of different systems; the ways in which they are activated is diverse and some systems are better understood than others.

Congrats on a nice study.

Referee #1:

In the manuscript "Bacteria elicit a phage tolerance response subsequent to infection of their neighbors" Tzipilevich and Pollak-Fiyaksel et al. investigate the phenomenon of "shrinking plaques" over the course of phage infection in solid medium. The authors demonstrate that Bacillus subtilis displays transient resistance to infection by the phages SSP1 and Phi29. Importantly, while highly divergent, both phages utilize wall teichoic acid (WTA) as receptors during infection. The authors define the transient resistance as "tolerance" and quantify this trait as the ability of the bacteria to invade into the zone of clearance created by the phage at approximately 8 hrs post infection, resulting in plaque size reduction. The authors go on to demonstrate that tolerance is mediated by the sigma factor SigX, which, upon phage infection, upregulates the dlt operon thereby resulting in modification of WTA and reduced phage binding/infection. Furthermore, when the authors engineer the bacteria to overexpress SigX prior to infection there is dramatic reduction in plaque formation, indicative of limited phage infection. The authors used a combination of genetics and microscopy to clearly demonstrate this phenomenon. This work is of good quality and the conclusions are mostly supported by the data. Below are a few points to help solidify conclusions reached.

We thank the Reviewer very much for the positive assessment of our study.

Major Comments

1. One of the principal findings from this manuscript is that a secreted factor from phage-infected cells activates SigX-dependent gene expression. These conclusions are reached from data presented in Fig. 3 and S5, however, those experiments lack an important control. In addition to uninfected PY79 + BS12, the GFP signal from BS12 should be analyzed when BS12 is co-cultured with ΔyueB +/- phage infection. If infected bacteria produce a diffusible signal that activates SigX, then addition of phage to a ΔyueB + BS12 co-culture should have no effect of BS12 GFP production. This experiment rules out a competing hypothesis that a component of the phage lysate used for infection is responsible for activating SigX.

To address the Reviewer's concern, we added phages directly to BS12 cells and measure GFP fluorescence. Of note, the addition of a Δ*yueB* strain was not required, as BS12 carries the Δ*yueB* allele. The results, showing no significant increase in fluorescence following phage addition to resistant bacteria, are now presented in Fig EV3D.

2. The fluorescent images of full plaques, such as those presented in Fig. 1E, are very impressive and a large part of the results put forward by the authors. However, these images could be greatly improved by expanding the field of view (data that may already be available). At present, it appears the entire plate overlay becomes dominated by WT at 16 hours post infection. However, based on the authors conclusions, only the phage-adjacent bacteria invading the plaque should be WT and the surrounding bacteria should remain an equal mixture of WT and SigX. Can this be shown to justify the authors conclusions?

The images of the plaques were captured at low magnification and, in general, the entire field is shown in the majority of the panels. The original images are now deposit as "raw data" (e.g. Fig 2E). Furthermore, to substantiate our conclusion, we provide

additional images of fields, taken away from the plaque area, whereby the two strains, WT and Δ*sigX*, appear to be distributed similarly, as corroborated by quantification analysis (Fig EV2$_{4,5}$). This is now also highlighted in text (p7, L10-11). Finally, we now show that Δ*sigX* cells have no growth deficiency when co-cultured with WT cells (see below).

If not, an alternative explanation for the observations in this manuscript could be that ΔsigX displays a growth disadvantage only when co-cultured with WT.
At present, the growth rates of WT and ΔsigX are investigated in monoculture, but the phage infections are in competition. A more appropriate comparison would be demonstrating there is no competitive defect for ΔsigX in co-culture.

To address the Reviewer's concern, we mixed GFP-labeled Δ*sigX* cells with either non-labeled WT or non-labeled Δ*sigX* cells. GFP levels indicated similar growth kinetics for both cultures, suggesting no significant competitive defect for Δ*sigX* in a co-culture. These results are now presented in Appendix Fig S2C, and referred to in the text (p7, L11-12).

3. Can the authors better explain the SPP-lysin-yfp strain and results? This reviewer's understanding of lysin proteins is that they are secreted and do not mark the phage virion but rather the remnants of phage infection. If that is the case, could data in Fig. S1 and Movie S3 suggest that the rim of the plaque is an area of active replication instead of fully formed phage virions unable to infect sigX expressing cells? It is conspicuous that the YFP signal in Fig. S1 does not appear to constrict yet is empty in the center of the plaque. These possibilities could be accounted for by altering the language in Page 5 Line 22 from "signifying that bacteria at the rim could withstand the presence of phages" to something that accounts for the decreased phage infection kinetics observed later in the manuscript.

Thank you, this was indeed unclear; we thus modified the text to indicate that the YFP represents cells undergoing infection (p5, L18-24), as described in our previous publication (Tzipilevich et al., 2017), and shown again in this manuscript (e.g. Fig 4B). As for the YFP rim, we do see that it constricts, and we added measurements to highlight the fluorescence rim diameter (Fig EV1C).

Minor Comments

1. In general, there is a lack of expected details and genetic controls presented. For example, many mutations appear to not be complemented in this study. Are fluorescent protein fusions of SigX functional? The authors should try to add these and other details.

To address the Reviewer's concern, we added data showing complementation of Δ*sigX* (Appendix Fig S1D; p6, L21-22), and tested the functionality of SigX-GFP (Appendix Fig S1E; p8, L 9-10). Details were added in the text. Methods and Figure Legends were revised to include complete experimental details.

2. The x-axis of many graphs and the timepoints of images shown are inconsistent throughout the manuscript and make comparisons difficult. For example, Fig. 2D, 4A,

5A (I think this should be min not hr?), S5C, and S7A all plot similar experiments but often have different x-axis ranges. Can these and other graphs be standardized?

In general, plaque formation experiments were followed for 15-20 hrs, whereas liquid infection experiments were typically followed in course of 2-4 hrs. Therefore for the later experiments the time scale is presented in minutes. We now attempted to fit the time scales in the presented panels.
Thank you, 5A should be in minutes and was corrected.

3. Fig. 3A x-axis spelling error
Thank you, corrected.

4. This reviewer found the transition to why extracellular sigma factors were investigated difficult to follow. Further, I was disappointed in the lack of discussion for other SigX-regulated genes, such as ywbO. While not critical, if there is sufficient space, I would appreciate some discussion here.

We now attempt to better explain the transition to testing the extra-cytoplasmic sigma (σ) factors (p6, L7-9), and refer to *ywbO* in the Results (p10, L14-16) and Discussion (p13, L22-25).

5. On page 7 line 9 the word "infection" and should say "infected"

The sentence was slightly modified (p7, L15-17).

6. For Figure S8B the labels on the two images are the same. This reviewer believes the images are meant to represent two different time points and thus the labels should be altered.

Thank you, corrected (now presented in Appendix Fig S3B).

Non-essential Suggestions
1. One additional note, but by no means a necessary requirement, have the authors considered treating B. subtilis with sub-lethal concentrations of antibiotics/antimicrobial peptides to induce SigX expression? Do these stimuli alter phage tolerance? This might be an exciting extension of the data presented. But again, this is outside of the scope of the current study and perhaps work that is already in progress.

Thank you for raising this interesting aspect. In fact, we think that that cross activation of SigX enables bacteria to simultaneously resist multiple stress conditions, similarly to the heat response we observed here (Discussion, p14 L5-10). Indeed, as the Reviewer commented, we are now further investigating the impact of antibiotics on the phage tolerance response.

Referee #2:

The work developed by Tzupilevich and colleagues is exciting and well designed. They bring techniques that will fuel novel discoveries within the group and others who investigate in this space. This manuscript explores bacteria's first line of defense against phages, which the authors point out as mostly unexplored and uncharacterized. With some of the community focusing on simply cataloguing new defense systems, studies like these are a breath of fresh air, getting us closer to the true ecological understanding of phage-host interactions. I would therefore like to congratulate the authors for this work. It was a pleasure to read, and therefore it is my pleasure to recommend this paper for publication after the authors address some minor recommendations. I understand that some of the suggestions are at the rim of this manuscript, some intended to stimulate discussion, others to improve the clarity to the reader.

We thank the Reviewer very much for the positive assessment of our research.

Recommendations:

1) The work focuses on studying phage-host interaction on solid media. When comparing the agarose pad results with the plate data it is unclear if the experiments are done in solid media or semi-solid (low agar percentage in double-layer agar technique). I recommend the authors to make this clearer in Material and Methods, and change the text if these are comparing semi-solid conditions with solid media;

All the plaque assays were carried out on solid medium, and did not use double-layer agar technique. We now indicate that clearly in the Material and Methods (p17, L22; p18, L2).

2) What is the number of phages in the center vs the rim of infection?

We attempted to address the Reviewer's question by sampling phages from the plaque center and rim. However, our attempts yielded very variable and inconsistent numbers making the quantification difficult. Of note, experiments with fluorescently labeled phage ($SPP1_{lysin\text{-}YFP}$), revealed a similar fluorescence level throughout the experiment at the plaque rim, suggesting significant numbers of phages at the plaque periphery during the constriction phase. These results are presented in Fig EV1A.

3) The authors report that the isolated bacteria from the rim are phage sensitive but data is not shown. I suggest adding this data to the manuscript;

These data are now presented in Fig EV1D.

4) Could the authors make it clearer in line 7-8 of page 6 that the hypothesis is linked to cell envelope stress?

This part was modified and is hopefully clearer now (p6, L6-9).

5) Do the authors know if phage replication is not affected at 48C? In Phi29, the burst size at 45C in a different strain has been shown to reduce from 570 to 260. What about in this strain for both phages?

To address this comment, we have carried out experiments to estimate PFU/ml at 37°C and 48°C. Indeed, we found a decrease in PFUs of both phages at 48°C (~30% reduction for both Phi29 and SPP1 in WT or Δ*sigX* hosts). However, the slower growth observed at 48°C could substantially contribute to this reduction. Since we are comparing plaque size (not numbers) of PY79 and Δ*sigX* strains, having a similar growth rate and infected with the same phages (Fig EV3A-EV3B), we think that the comparison is reasonable. Nevertheless, we toned down the text and added an appropriate reference regarding this issue (p7, L17-19).

6) I suggest that the authors revisit the word transcription on line 13 page 8. Perhaps the word 'expression' would be better suited to the observation of fluorescent signal measurements that are knowingly affected by protein turnover rates.

"Transcription" was replaced with "expression" (p8, L22).

Suggestions:

1) The two phages used in this study have different reported burst sizes: 200-250 and 570, SPP1 and Phi29, respectively. Can the authors comment on the potential effect on the constrain reported and phage spreading?

The issue of bust size is now included in the Discussion (p13, L6-9).

2) The authors suggest a new term: phage tolerance response. Considering that all isolated bacteria from the rim are phage sensitive, do the authors consider that the mechanism of sensing and spreading initiates a cascade of process leading to non-reversible mutations in the community, or that is more likely that the phage pressure at the rim will select for these mutants?

Thank you, this is indeed an important point that is now included in the Discussion (p14, L14-17).

3) The schematic Figure 3A and 3B should be improved to help the reader understand better the plotted results;

We have now improved the schematic in Fig 3A and 3B to make the experimental design clearer.

4) For clarity I would suggest that the authors would represent the individual measurements in Figure 4, 5, S5 and S6;

To address this Reviewer's concern, we have included all the individual measurements in the raw data for the listed figures in this submission.

5) Figure S7 does not seem color blind friendly and would be better if the authors would plot the average.

To improve this figure (now EV5A), all the mutants are now displayed in separate individual graphs.

Typo:
Please correct the word infection in Figure 3A.

Thank you, corrected.

Referee #3:
Tzipilevich et al. used fluorescent microscopy to monitor phage plaque dynamics over time in B. subtilis and two of its phages, SPP1 and phi29. They find that after plaques expand, they restrict in diameter, indicating bacterial growth back into the cleared zone. The re-taking of the plaque by bacteria was linked to the activity of the sigma factor sigX, which controls the dlt operon that cell wall teichoic acids (TA). A soluble but undefined molecule able to diffuse through a membrane induced sigX-dependent TA cell wall remodeling.

The authors mention that plaque formation dynamics are understudied, and it is true. I have considered it myself many times: why don't plaques continue to grow and over take the whole plate? This was an interesting study and I believe it important to the field of phage biology in general. I have only minor comments:

In general, red and green images were difficult to differentiate (I am color blind). –

We apologize to the Reviewer for not displaying our results in colorblind friendly scales originally. We have now modified all the results, including microscopy images, graphs, and movies into colorblind approachable colors.

Line 14, page 2: "propelled by transcriptional response" Are the authors referring to a phenotype here as opposed to a genotype?

Yes, to make this clearer we wrote: "propelled by a programed transcriptional response" (p4, L17).

Line 16-17, page 5: "...indicating that such a kinetic pattern is widespread." I suggest using a less definitive word (suggests instead of indicates) as only 2 phage species were tested, so it is difficult to claim something is very widespread.

Corrected (p5 L16-17).

Line 24, page 5 through line 1 page 6: "all tested bacteria remain phage sensitive." Please refer the reader to the data.

These data are now presented in Fig EV1D.

Fig 2A: more intuitive for readers (especially color-blind readers) if strain names were placed below the data rather than listed as a column to the side.

The figure was modified accordingly.

Line 14 page 6: Indicate to reader what conditions cells were grown when comparing wt and ΔsigX strains.

We now indicate that growth was measured in "liquid culture" (p6, L14-15).

Line 17 page 6: "No difference in lysis kinetics was monitored when bacteria were infected.' "Observed" preferred over "monitored"

Modified (p6, L19).

Line 9 page 7: Authors state there is no difference in growth at 48C. Can the authors please clarify that they are comparing wt and sigX strains at 48C as opposed to comparing growth at two different temperatures?

This is now clearly stated (p7, L17-18).

Line 8 page 9: More details on reversible vs irreversible phage attachment would be welcome.

The information was added (p9, L16-19).
Of note, this issue is also described in detail in the Introduction part (p3, L25-p4, L2).

Line 7-8, page 13: I am not sure I fully agree with the statement "it is not entirely understood how activation of the CRISPR-Cas system is prompted." CRISPR-Cas is a general term that encompasses a lot of different systems; the ways in which they are activated is diverse and some systems are better understood than others.

This was modified to say: "the various CRISPR-Cas systems" (p14, L24).

Congrats on a nice study.

Thank you very much for the positive assessment of our manuscript.

References

Tzipilevich E, Habusha M, Ben-Yehuda S (2017) Acquisition of phage sensitivity by bacteria through exchange of phage receptors. *Cell* 168: 186-199 e12

Dear Prof. Ben-Yehuda,

Thank you for the submission of your revised manuscript to The EMBO Journal. I have looked at your response to the referees and now consider that you have properly dealt with all of their major concerns. However, before we can proceed with the acceptance of your study, there are a few minor editorial issues that need your attention:

- You provide 7 keywords describing the topic of your paper and the maximum we allow is 5. Please remove two of them.
- The manuscript needs a Data Availability Section where you describe the databases in which datasets have been made available. For transparency, this section is required even if just to mention that there are no public datasets associated to the results in the paper.
- Movies must be provided in ZIP folders including a text document with their legend (currently included in the manuscript). Each movie and its corresponding legend in a separate ZIP folder.
- Correct nomenclature for Appendix figures and tables is "Appendix Figure S1" or "Appendix Table S1" respectively. Please correct the file and make sure all callouts in the manuscript are modified accordingly.
- Source data must be presented as one file per figure. For Excel files, different tabs can be used for the different panels (A, B, C). For figures including source data in different formats (Figures 2, 5, EV2), a folder containing the Excel and the PDFs files must be provided.
- The paper's synopsis is composed of:
1) A short 'blurb' text summarizing in two sentences the study (max. 250 characters) and three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format. This text has not been provided.
2) A "synopsis image", which can be used as a "visual title" for the synopsis section of your paper. While you provide this image, it needs to be resized. This figure must be in PNG or JPG format with pixel dimensions of 550 x 300-600 (width x height).

Please let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal and congratulations!

I look forward to receiving the final version of your manuscript with these minor changes included.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Please click on the link below to submit the revision online:

Link Not Available

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision **12th Nov 2021**

Dear Prof. Ben-Yehuda,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
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Yours sincerely,

David del Alamo
Editor
The EMBO Journal

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Corresponding Author Name: Sigal Ben-Yehuda
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2021-109247

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:

a specification of the experimental system investigated (eg cell line, species name).
the assay(s) and method(s) used to carry out the reported observations and measurements
an explicit mention of the biological and chemical entity(ies) that are being measured.
an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

the exact sample size (n) for each experimental group/condition, given as a number, not a range;
a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
a statement of how many times the experiment shown was independently replicated in the laboratory.
definitions of statistical methods and measures:

- common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
- are tests one-sided or two-sided?
- are there adjustments for multiple comparisons?
- exact statistical test results, e.g., P values = x but not P values < x;
- definition of 'center values' as median or average;
- definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	in general at least 3 different biological repeat were conducted.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NO
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5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We use excel to analyse the data
Is there an estimate of variation within each group of data?	Yes

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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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G- Dual use research of concern

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