nature portfolio

Peer Review File

Pseudomonas aeruginosa faces a fitness trade-off between mucosal colonization and antibiotic tolerance during airway infections

Corresponding Author: Professor Alexandre Persat

Version 0:

Decision Letter:

8th April 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 Alexandre,

Thank you very much for your patience while your manuscript "Pseudomonas aeruginosa faces a fitness trade-off between mucosal colonization and antibiotic tolerance during infections." was under peer-review at Nature Microbiology. NMy apologies for the time taken to get news to you. Your manuscript has now been seen by 4 referees, whose expertise and comments you will find at the end of this email. They find your work of potential interest, however 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, you will see that several concerns were raised as to whether pre-selection might have occurred during the preparation of the Tnseq library inoculum and how this might have affected interpretation of the results. There were also several points raised regarding the need for further controls to validate findings pertaining to antibiotic tolerance and cross-protection and to rule out involvement of other, non-EPS cyclic di-GMP-regulated responses in the phenotypes observed. In addition, there were requests for further information on the computational methods used for the metabolic modelling and tSNE analysis, to address discrepancies between simulation data and experimental results, and whether further data is available that could support the conclusions regarding ornithine, arginine and proline. Furthermore, there were several requests from Reviewer #3 to validate several features of the model systems, and to clarify the strengths and weaknesses of each and when/why they were used. These are points which we feel are critical to be addressed for us to consider a revised manuscript. The remaining issues outlined in the referees' reports, should be clear and straightforward to address. Although Reviewer #2 recommended editing the methods and moving information to supplementary, please keep all methods in the main manuscript file but consider working with the text to make sure that all methods are explained in full and easy for researchers to understand and potentially implement.

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

If revising your manuscript:

* 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.

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* 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.
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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.

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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,

[redacted]

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Reviewer Expertise:

Referee #1:
Referee #2:
Referee #3:

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

Overall comments

• This paper is impressive, with a wide range of techniques used to provide multiple, independent layers of evidence, along with appropriate statistical tests and visualisation to demonstrate the key findings. A good read
• Supplemental files provide good additional information.
• There could be more clarity surrounding the metabolic modelling aspects of the paper, though it does not hinge on these data.

Introduction

• L39-40: Awkward phrasing of “we still know little...”. Consider revising to “little is known about...”
• L59-62: Nice. Good justification

Results

• Fig 1 and S1: Good clear figures. Nice job
• L110-112: Cool experiment
• L113-117: Interesting findings.
• L114: I would remove ‘very’. 0.7 = strong correlation, don’t need adverb. Would also add “(r = 0.74)” to this line
• L127-129: Did the single gene knockout analysis for metabolic modelling also indicate this, when either lactate/glucose used as a sole carbon source?
• L132-135: Great follow up experiment here.
• L148-149: Were ornithine, proline, and arginine present in the simulated SCFM? If so, you could also test this by removing these from the SCFM media? Linked to this - Fig 2C: I am wondering why the SCFM is so considerably different to the CF results in Fig 2C. What is the authors explanation for this discrepancy, which is essentially a lot of false positives in the SCFM modelling here. Does this indicate that the modelling is not all that accurate for these growth conditions?
• Fig 2D: It is not clear to me what the t-SNE is actually comparing here. Is it the result of growth under various media conditions for each single gene knockouts? This information is also absent in the methods section and could do with clarification. Also, could you explain how this ‘circle’ was drawn? It seems arbitrary - was there any specific thresholds or clustering algorithms used? I don’t disagree with your general findings, as Orn, Pro and Arg are closer to your Healthy and CF points, but a systematic explanation/analysis is preferable here. Also, is there any MS data published which shows Orn, Pro and Arg being prevalent in the mucosa specifically, aside from “dead host cells, which can constitute a pool of amino acids”? This would be a useful reference if it exists
• L155-157: To clarify, clustered based on what? What output was being measured here and clustered? I don’t necessarily disagree with the summary in 158-159, but this needs further clarification.
• L170-173: I really appreciate your LB media controls here and previous experiments, as it really demonstrates the importance of environmental context for genes and their impact on fitness.
• L201-214: Really great work here. 4D-G is nice. Also, great work with the cluster area methodology
• L241: Do you mean 5A?. L249: Do you mean 5B?
• L267-269: Agreed. Very nice experiments here. Do you have a no antibiotic control?
• L286-296: Really nice work with this, which shows the effect very clearly. Not sure if authors have considered this, but do planktonic cells provide any benefits to biofilm-forming cells? Or is it more of a commensalistic or even parasitic relationship? I am thinking of your point about the biofilms in L232 regarding their impact in: “maintain[ing] local host tissue integrity”. The depletion of epithelial cells in large biofilm (6C, last panel of top row) shows considerable depletion of epithelial cells. Is this actually negative for the biofilm-forming cells and thus, deleterious to the overall community? Not recommending any experiments or anything here. But say, comparing these results to S3 B at t = 10 h, I wonder if the impact on host epithelial cells and thus attachment for biofilm causes an overall loss in biofilm? Maybe it’s as you say though - “phenotypic heterogeneity and flexibility”.

Discussion

• Each statement is well justified, referenced and backed up by evidence in results section.

Methods

• Methods are robust in general but more detail for computational methods is required
• L629-630: What version of TPP and BWA MEM?
• L669-671: So if there was hypothetical/genes of unknown function, were they just not functionally classified, or were they were left out of additional analysis? If so, that doesn’t seem right to me - highlighting these could lead to additional focused studies in the future where their function could be determined. A lot of the highest and lowest logfold change genes in the supplementary tables are hypothetical genes. I’d recommend adding an ‘Unknown function’ bar to 1C. It’s important to highlight how much knowledge we are lacking.
• L684-685: What versions, what commands?
• For Transwells, what volume was added to well and to Transwell itself? Just the standard 600 µL into the well and 100 µL into the Transwell?
• L776-777: How? What tool/commands were run? If pyTFA, what version?
• L779-781: “We used the thermodynamically curated to assess…” should be “We used the thermodynamically curated model to assess…”? So do you mean that 40% of the encoded reactions in the PAO1 model were not used in analysis of growth in different conditions? If so, this may be an oversight by not considering genes from a well validated model. This may partially explain the differences seen in the simulated CF vs real CF results.
• L781-786: Could the authors please also provide the ingredient list for their simulated growth medias in Supp, Figshare or something equivalent? Ie what constitutes the ‘minimal media’? Also, what packages and commands were used to perform these simulations? Ie cobrapy for single gene knockout?
• L802: What was the input data for t-SNE?

Reviewer #2 (Remarks to the Author):

In this manuscript, Meirelles et al. present a very interesting study based on a massively parallel sequencing approach of a Pseudomonas aeruginosa Tn mutant library pool grown on the mucosal surface of human primary bronchial epithelial (HBE) cells in ALI cultures and in an advanced organoid model (AirGels). This study implicates that biofilm formation is a fitness burden for bacteria during mucosal colonization (no additional selective pressure), whereas this burden changes into a fitness advantage under antibiotic stress. Furthermore, the authors show that hyper-biofilm formation not only confers antibiotic tolerance to the mutant cells themselves, but also cross-protects WT-like single cells in close proximity.

The combination of HBEs from healthy and CF donors in two different culture models (ALI and AirGel organoids) together with a PAO1 Tn mutant library is a novel approach to identify determinants of bacterial fitness on the established mucus surface (up to 3 months). Surprisingly, typical biofilm determinants, which are a hallmark of bacterial adaptation to chronic and persistent infections, showed a reduced fitness. The authors suggest that local nutrient limitation (due to limited exploratory behavior) reduces fitness of hyper-biofilm formers, but confers a selective advantage when bacteria are challenged with antibiotics.

I would like to emphasize and acknowledge that the authors have carried out a whole series of very lengthy and complex experiments to gain a more comprehensive understanding of P. aeruginosa adaptation during infection.

Major comments:

1) My main question is: What are the mechanisms behind the observed increased tolerance and the cross-protection of planktonic WT cells co-colonized with large aggregates of the hyper-biofilm former ΔwspF?
It is certainly a very interesting observation that biofilm producers only have an advantage under selective antibiotic stress conditions. Nevertheless, there are countless reasons that could lead to this tolerance (reduced penetration of the antibiotic, gradient of nutrients and oxygen etc.). Do you have a hypothesis?

2) In the first part of the study, the authors use a HBE-ALI model, while in the course of the study they switch to a complex organoid model (AirGel). For some experiments healthy donor cells were used, for others cells from CF donors. It is a bit hard to follow and I would appreciate if the authors would make it clearer which model and cell line was used for a specific experiment (and why).
Please include the specific advantages of the AirGel vs. ALI cultures and point out similarities/differences. Are the models interchangeable? What differences could you find in the Tn-seq output pools between ALI and AirGels?
Also, for further clarification, I would suggest that the authors adjust their simplified representations in each figure so that it is obvious for the reader, which particular model and cell line was used. For example:
- Adding a schematic to Fig. 3
- Adjusting the schematic in Fig. 6 (AirGel; similar to Fig. S3)

3) The experimental design of the Tn-seq approach seem to have some weaknesses:
- Tn libraries were grown for 14h into deep stationary phase before being administered to HBE cultures (Line 541 ff). This already selects for and at the same time against certain mutants. I wonder how many genes of the PAO1 genome were actually covered by the inoculum used? Was there a bias within the inoculum towards mutants that have a fitness advantage in nutrient- and oxygen-limited conditions (as encountered in deep stationary, but also in biofilm conditions)? How many genes are enriched/depleted in the inoculum pool compared to the original Tn library?
- The exposure times to the antibiotics were kept very short, followed by a long outgrowth step of 7-12h (Line 573ff). Does the population composition of the output pool reflect differences in fitness as a response to the respective antibiotic or rather differences in (re-)growth capacity? (e.g. due to shorter lag times, higher doubling times…)
As far as I understood, this study was based on one replicate per condition. Independent replicates would potentially have helped to identify noise and address my above-mentioned concerns

4) The focus of this study is on the polysaccharides Pel and Psl and their regulation through c-di-GMP signaling, which also involves the Wsp system. However, I am missing the third polysaccharide alginate. The overproduction of alginate leads to mucoidity and is a hallmark of Pa adaptation during chronic infections. In addition, alginate production is also regulated by c-di-GMP (e.g. https://doi.org/10.1111/j.1365-2958.2007.05817.x; https://doi.org/10.1128/spectrum.00675-22). In particular, algR regulates c-di-GMP levels and biofilm formation through the diguanylate cyclase mucR (e.g. https://doi.org/10.1093/nar/gkv747). Both, algR and the related gene mucA, were identified as hits in some Tn-seq conditions. Therefore, the existence of alginate should at least be recognized.
Along these lines, there was a recurring pattern, namely that loss of motility (e.g. flg and pil genes) increased fitness in mucosal colonization, but decreased fitness under antibiotic treatment. Similar to e.g. hyper-biofilm formers, these strains have limited exploratory properties (but due to completely different mechanisms).

5) I am lacking some additional control experiments and information:
- Regarding antibiotic treatment: Are there differences in the MICs for CIP/TOB between e.g. ΔbifA or ΔwspF and PAO1 WT? This would untangle whether the observed increased survival is due to resistance or tolerance.
- Was the tolerance only confirmed by fluorescence measurements or also by CFU counts? (e.g. Fig. 5)
- Were control experiments with PAO1 overexpressing pel and/or psl performed? This would further confirm that other cdG-regulated traits make only a minor contribution to the observed phenotypes.

6) In general, all parts of the methods section feel very lengthy, somewhat unstructured, and many passages seem redundant. All in all, this makes it a bit difficult to find the relevant information. Please shorten wherever possible and consider moving parts to the supplements.

Additional comments:

Abstract:
Line 25: Wording. Repetition of limited/limits.
Line 27: specify which biophysical mechanisms you are referring to
Line 28: I feel like the term “genotypic heterogeneity” is not entirely correct here. Different mutations can lead to the same phenotype (antibiotic tolerance in this case), meaning that a genotypically heterogeneous population can indeed be phenotypically homogeneous (tolerant) without fitness costs. To emphasize the fact that susceptible cells are protected, I would rather suggest to use a term like: “population survival”, “phenotypic heterogeneity”, etc.

Introduction:
Line 47: Wording. Since the transition from acute to chronic (and back) can be a reversible, I would suggest to use something like “strains with an acute lifestyle”, “strains that have adopted an acute lifestyle”, etc. instead of “acute strains”. The same applies to the section starting at lines 276ff)

Lines 52 ff: Although it plays a minor role in the type strain PAO1, the existence of alginate should at least be mentioned (see point 4).

Line 54: Introduce the abbreviation cdGMP when mentioning cyclic-di-GMP for the first time
Line 56: You might want to add the following reference “Evolution of Antibiotic Tolerance Shapes Resistance Development in Chronic Pseudomonas aeruginosa Infections” (https://doi.org/10.1128/mbio.03482-20)

Line 74: Wording. “Mimic” instead of emulate (see also line 304)
Line 76: Wording. “do not take into account” or “do not consider” instead of overlook
Line 83: Wording. I personally think that the term “functional genomics” is a bit exaggerated for a Tn-seq approach, as the phenotypic outcome is only assessed at the population level (mutant pool) and not for each individual mutant. I would appreciate if the term "functional genomics" would be replaced by "high-throughput massively parallel sequencing approach" or something along those lines. The same applies to lines 90, 364, 239.

Line 83: Wording. Repetition of tracking/track

Results
Line 93: Move the reference so that it appears directly after Tn-seq
Line 95: Wording. “determined” instead of considered?
Line 99: Wording. “mutants that were selected” or “mutants with altered fitness” instead of selected mutants

Line 103: Please add how many of the 620 genes were enriched/depleted. Which log2FC threshold did you use to identify significantly enriched/depleted mutants?

Line 112: add the significance threshold used (adj. p val and log2FC)
Line 120: Wording. “thrives in” instead of thrive to
Line 123: Add that you look at both healthy and CF HBE cultures.
Line 130: Wording. “produce” instead of make
Line 153: Add reference for sputum composition / synthetic sputum medium
Line 156: Wording. clustered with our “experimental” data most closely
Line 161: Wording. “penalty” is in my opinion a bit too bold. Please rephrase (see also lines 268; 398)
Line 174: Wording. “sessile” instead of sedentary? (see also line 219)
Line 178: Does the ratio of the two competing strains have an influence on the colonization pattern? Did you test for other ratios? I could imagine that they are not necessarily co-existing 1:1 in the natural environment.

Line 185: Briefly mention why it is advantageous to switch to AirGels. What do this model provide that is not covered by the previously used HBE cultures? (see point 2)

Line 187: To me, it looks like continuous slow growth of ΔwspF throughout the experiment with a rather constant low growth rate. Please provide numbers to indicate the two different growth phases of ΔwspF (or rephrase)

Line 198: Wording. “forced to stay” implies outer forces. It’s more correct to state that the ΔwspF mutant (as well as the ΔbifA mutant) just do not have the capability to explore and disseminate (high cdG reduces motility). Please rephrase.

Line 220: Please clarify whether you have used healthy or CF HBE (see point 2)
Line 221: Wording. Although 11h of infection is quite something for a virulent strain like PAO1, I would not call it “long-term” infection

Line 231: Wording. Specify under which conditions growth is impacted: Overall, while biofilm formation negatively impacts P. aeruginosa growth “during mucosal colonization” by limiting access to nutrients…

Line 237: Wording. The header might imply that there is some tolerance towards colonization. Maybe better: “Colonization vs. tolerance trade-off…” or “Trade-off between colonization and tolerance for biofilms during antimicrobial treatment”. This also

applies to Line 443.

Lines 237ff: The text refers to Fig. 6x, although this section is about Fig. 5. Please correct the entire section.
Line 239: Please state whether you have used healthy or CF HBE
Line 241: Please include the antibiotic concentrations relative to the MIC (e.g. 10x MIC). This also applies to lines 263; 283.

Line 248: Wording. Re-order sentence
Line 251: Wording. Have any clean mutants with fitness defects during mucosal colonization actually been tested for their (biofilm) tolerance to ciprofloxacin? If not, please rephrase, e.g. “Several mutants displaying fitness defects during mucosal colonization showed increased fitness during ciprofloxacin treatment”

Line 255: Wording. Tolerance of the mutants was not tested (see above)
Lines 255ff: Please provide a separate list or mark the 24 hits that were found for both TOB and CIP-treated biofilms in Table S4. Mention in the text that some of the selected cdG-related example genes (displayed in Fig. 5C) were significant in only one of the two treatments. Also, I have difficulties finding dipA in Table S4. Please check.

Line 260: Wording. “To better assess” or “To make accessible” instead of expose
Line 266: Reduced biomass could possibly be an indicator of killing. Was the killing additionally quantified using PI staining (expecting an increase in dead cells over time)? Or CFU counts?

Line 277: Wording. “genetic” diversification
Line 278/280: Wording. Better: “strains displaying characteristics of planktonic (acute) and biofilm (chronic) lifestyle” or “strains adapted to chronic infection”

Line 280: Doesn't the definition of planktonic (free-living single cells) exclude the possibility of these cells being in a biofilm? Please specify or rephrase.

Line 282: Why have you used ΔwspF although it was not amongst the hits of the Tn-seq experiment (CIP treatment)? Have you checked if ΔbifA shows similar cross-protection of WT cells as ΔwspF? Also, it is surprising that ΔwspF cross-protects, but didn’t show up as a hit in the Tn-seq experiment.

Line 287: I assume that “rapid killing” indicates that planktonic cells and small clusters were already dead 4h post CIP addition. It would be helpful to additionally show images from T0 (when the drug was administered) to illustrate this rapid killing (or define in which time frame “rapid killing” happened)

Line 293: see above (line 280)

Discussion
Line 305: What is the difference in “chemical composition” of AirGels and ALI? Why are AirGels better in mimicking the chemical composition of the in vivo situation? Please specify or rephrase.
Line 335: Wording. “mutational hot spots” instead of evolution
Line 352: Wording. “massively parallel sequencing” or “Tn-seq” instead of “omics-based methods” (only one technique was applied)

Figures
Fig. 1
Panel 1C: Since your work focusses strongly on genes involved in biofilm formation and cdGMP signaling, I would suggest to perform a functional enrichment analysis based on GO terms. This would be much more meaningful and supportive than PseudoCAP functional categories, which are quite broad. Additionally, since functional groups are very variable in the number of genes they contain, the representation as counts may be misleading. Please display the enrichment as % or fold-change enrichment or include at least the number of genes within each category.
Panel 1D: I suggest to highlight mutants involved in biofilm production in a different color to display their location on the correlation plot

Fig. 2
Panel 2B: I would find it more accurate to show growth as number of generations/8 hours.
Line 380: add “…compared to the LB control”. This also applies to line 400.
Line 385: Wording. “the red square indicates simulated growth”
Line 389: Are the conditions within the circle manually selected by eye or has a statistical approach been used? (e.g. 95% confidence intervals) Please specify.

Fig. 3
Panel 3C: An additional merged projection would be nice to display colocalization of WT and the competing strain
Line 404/412: unify 1 to 1 and 1:1

Fig. 4
Panel 4B/G: Could a histogram be more informative than the shown cumulative distribution?
Line 426/427: integrate the cdGMP part from the sub header of panel D into the main header

Fig. 5

Panel F: Why does the fluorescence start at 0 (when CIP is added) and peak after approx. 5h? (see also Fig. 6E)
Some of the ΔbifA replicates seem to resemble the WT patterns quite closely. Is this due to differences in aggregate size? Or what could be the reasons?
Line 453: Clarify what was quantified here (living cells)

Fig. 6
Line 464: Wording. “created” hints towards an active process. To make this statement, it would be necessary to show the same area before treatment.

Methods

Line 484: I assume CIP was further diluted in water?
Line 573: How were the optimal antibiotic exposure times determined/selected? Why was the antibiotic exposure time longer for TOB than for CIP?
Line 658: log2 fold change (instead of chain)
Lines 687 ff: Consider moving the section about cultivation of the HBEs to the beginning (e.g. directly before you describe the Tn-seq experiment in lines 536 ff).
Lines 757 ff: Shorten and mention only relevant modifications to the previous paragraph
Line 779: A noun is missing “thermodynamically curated …”
Line 708: explain what PDMS stands for
Line 815: Were the main cultures grown in presence or absence of gentamicin?
Line 854: How was the “small cell size” defined?
Lines 876 ff: Consider moving the section about imaging of bacteria directly before you describe competition experiments (lines 809 ff) and image analysis (lines 834 ff)
Line 905: Are the two fluorophores used in this study comparable to each other in terms of stability and metabolic requirements to produce the fluorophore etc.? I am wondering if a control experiment was performed to prove that the observed differences in fluorescence were indeed due to altered growth und not due to biochemical differences of the fluorophores (e.g. by swapping the fluorophores)
Line 907: “Tamara’s”?
Lines 952 ff: Which antibiotic concentrations were used? What are the MICs of the strains tested? (see point 5)

Supplements

Fig. S1: Add relevant experimental details (e.g. about the bacterial inoculum [OD600; CFU; MOI…], duration of infection…) to panel B and/or the figure capture

Fig. S3
A: AirGels were infected “with” WT or…

Fig. S4
- Wording, not all genes identified encode regulators. Maybe better: Tn-seq analysis identifies “genetic determinants” of antibiotic adaptation at the mucosal surface
- Outgrowth for how long?

Supplementary table S1:
- It would be helpful if you could add the functional categories to all genes and mark hits that you consider as being associated with biofilm formation/cdGMP signaling
- What do Sum Ctrl, Sum Exo, Delta Mean represent? I cannot find an explanation

Supplementary table S3:
- Add mexZ to the respective PAO1 locus ID

General:
- Unify healthy / non-CF HBE throughout the manuscript (e.g. Fig. 1 compared to Fig. 2/3)

Reviewer #3 (Remarks to the Author):

In this manuscript, the human airway mucosal surface was emulated leveraging tissue-engineered patient-derived airway organoids, which could mimic the mucociliary clearance function of the airway epithelium. With this organoid platform and the Tn-seq-based methods, the metabolic and dynamic mechanisms by which biofilm-dwelling P. aeruginosa experienced fitness trade-offs to adapt to the mucosal pressures and tolerate antibiotic treatment to initiate chronic infection were elucidated.

Overall, the study is well designed, clearly written, and represents a significant amount of work using very complimentary approaches and techniques. The authors presented interesting and important findings; however, I believe the following should be addressed before the manuscript can be accepted:

Major comments:
1. Since airway organoids were used in this manuscript as a novel model to replicate the biophysical features of the airway environment, the typical features of the airway organoids should be clearly characterized to demonstrate its similarity to the actual airway epithelium, especially the cell composition and mucus secreting function. For example, to identify the ciliated columnar cells, secretory cells, etc. by fluorescent immunostainings.

2. Expected as a bridge linking the gap between traditional “test tube” models and clinical studies, the organoid model facilitated with AirGels should be analyzed on its mucus composition by mass spectrum, especially for a comparison of the secreted mucus composition between the HBEs derived from CF vs. non-CF patients.

3. Two types of airway organoid models were applied, including HBE cultures on Transwells and AirGels. Please describe the similarities and differences between these two models and explain the reasons for switching between them. Please convince the readers the advantages of AirGels over Transwells.

4. The final step of AirGels chip fabrication was transition from immersed condition to ALI condition, in order to produce a lumen filled with air. Please provide evidences, such as cross-sectioned images of AirGels, to demonstrate the air-filled lumen and clearly show its dimensions, so that similarities between the model and the actual bronchioles can be visualized.

Minor comments:
1. Line 106-107: “Also, we found a surprisingly strong selection against mutants in genes coding for biofilm formation and cdGMP signaling (Fig. 1C, Table S1).”
Please list the terms in Fig.1C related to biofilm formation and cdGMP signaling.

2. Line 115-117: “In particular, biofilm production was selected against in the CF HBE cultures despite their clinical dominance during chronic infections.”
Please provide data supporting this conclusion.

3. Line 248: “We found a mild negative correlation between fitness mucosal growth fitness.”
Is the first “fitness” redundant?

4. Fig1.D suggested CF and non-CF HBE cultures imposed similar selective pressures on P. aeruginosa. However, in clinical setting, CF patients much more frequently have extensive P. aeruginosa colonization in the respiratory tract. Please illustrate the possible reasons why there is no difference shown on the performance of P. aeruginosa in the current experiment settings employing CF vs. non-CF HBE cultures. Analysis on the mucus composition may shed more light on this issue.

5. R2 is a negative value in Fig5.B correlation growth vs TOB. Is it an adjusted R2? Please explain its statistical validity.

Reviewer #4 (Remarks to the Author):

The manuscript “Pseudomonas aeruginosa faces a fitness trade-off between mucosal colonization and antibiotic tolerance during infections” describes a TnSeq analysis of P. aeruginosa colonizing organoids that the investigators developed, and termed AirGels. The Airgels are “a tissue-engineered 3D human lung organoid model that replicates biophysical features of the airway environment and that is suitable for high resolution live microscopy”. The investigators developed AirGels from epithelial cells and from human cells derived from cystic fibrosis patients. In TnSeq experiments, the AirGel organoids were colonized with a transposon library of P. aeruginosa, in order to identify genes with transposon insertions that caused reduced (or enhanced) fitness when cultured on the organoids, compared to other laboratory conditions. From the TnSeq analysis results, the investigators developed mutant strains with gene deletions, and performed imaging studies of the bacteria colonizing the AirGels, as well as competition studies, where the mutant strains were competed with wildtype to assess the sequence of events that occur during mucosal colonization by P. aeruginosa. They also used their TnSeq results to perform metabolic modeling and determine the nutritional requirements of P. aeruginosa when growing on the mucosal surface. Finally, the investigators performed a similar TnSeq experiment with P. aeruginosa cultured on AirGels and exposed to antibiotics tobramycin and ciprofloxacin. From their TnSeq results, the investigators focused primarily on genes involved in use of nutrients required for growth on mucosal surfaces and genes involved in biofilm formation, including polysaccharide biosynthetic genes and regulators of biofilm formation including genes involved in cellular cyclic-di_GMP levels.

Overall, I found this paper to be novel and interesting in that it describes the lifestyle of P. aeruginosa as it colonizes human derived mucosal surfaces. The investigators describe genes important in mucosal colonization as well as genes involved in “exploratory” behaviors as the bacteria move to new nutrient sources on the mucosal surface.

My primary critique is that the text of the manuscript is often vague, and that the supplemental movies (while cool) are often difficult to interpret. For example: when the cells look like they are completely covering the surface, how is the reader to interpret the difference between the behavior of the mutant and the wild-type strain. For example:

Line 187: When competing with WT, ΔwspF displayed two successive phases of colonization (Fig. 3F). In an initial phase ΔwspF grew exponentially, yet with a lower rate than WT. Later (> 9 h), ΔwspF growth stalled, and WT grew dramatically faster. At this stage, ΔwspF formed large biofilms that could not efficiently disperse, whereas WT was able to colonize new environments (Movie S1).

I’m not really sure how to interpret Movie S1, to show that colonization occurred in two phases. A clearer description of how to interpret the data in the text of the manuscript would be useful. The same is true for the other figures. For example, please

provide a better description of how cell death of both the mucosal cells, as well as the bacterial cells is interpreted. When describing the movies, it might be useful to include the statistics in the text, particularly when describing the differences between wild-type and mutant cells, as well as in the competition experiments. This would clarify the interpretation of the videos.

Minor points.
P. aeruginosa produces three different polysaccharides: Psl, Pel, and alginate. Alginate is not mentioned anywhere in the text. (although the strain used here, Pao1, is primarily a Psl producing strain). It can be induced to produce alginate or Pel, but it generally only produces Psl. CF Clinical isolates usually produce alginate.

The discussion section is short, and does not put the polysaccharide experiments in contexts of the literature (psl is not mentioned in the discussion. I think it could be expanded upon).

A better description of wspF and bifA would be useful. When I looked up wspF, it is described on pseudomonas.com as "probably methylesterase". So, unless the reader is really familiar with the Pseudomonas literature, it is not clear how a methylesterase would be involved in the overexpression of ci-di-GMP.

****

Version 1:

Decision Letter:

Our ref: NMICROBIOL-24010138A

25th July 2024

Dear Alex,

Thank you for submitting your revised manuscript "Pseudomonas aeruginosa faces a fitness trade-off between mucosal colonization and antibiotic tolerance during infections." (NMICROBIOL-24010138A). 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 two weeks. 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,

[redacted]

Reviewer #1 (Remarks to the Author):

The authors have appropriately addressed all my comments. Great work!

Reviewer #2:
Remarks to the Author:
I would like to thank the authors for their thorough and detailed response to the concerns raised. I greatly appreciate that the authors have now included a variety of additional control experiments that further validate and support their findings regarding biofilm tolerance (e.g. cfu counts, MICs etc.). My points have been answered in detail, both in the rebuttal letter and in the newly added or revised text passages/figures in the manuscript. In my opinion, the adjustments contribute significantly to clarity and transparency and make it easy for the reader to follow.
All in all, this study is of great importance and relevance to the field, and I congratulate the authors to this excellent work!

I have only a few very minor comments/suggestions:
Line 87ff: Grammar. "For example, AirGels are tissue-engineered 3D human lung organoids that replicate biophysical features of

the airway environment, and that are suitable for high-resolution live microscopy”
Line 105: It could be an idea to mention here that this work is based on PAO1 (since the Pseudomonas aeruginosa community mainly uses either PAO1 or PA14)
Line 604: Typo. “throughput” (instead of throughout)
Line 746: Reference to Table S5 is not correct here.
Lines 903, 978, 980…: Typo. “gentamicin” (instead of gentamycin)

Reviewer #3:
Remarks to the Author:
Thank you for your detailed responses. All my concerns have been addressed satisfactorily.

Liang Li, Ph.D.
Assistant Professor
School of Medicine
Southern University of Science and Technology
Shenzhen, Guangdong, China

Reviewer #4:
Remarks to the Author:
The manuscript "Pseudomonas aeruginosa faces a fitness trade-off between mucosal colonization and antibiotic tolerance during infections." is a revised version of a manuscript that I reviewed previously. The investigators used a combination of TnSeq, Airgel Organoids, and live cell imaging to understand biofilm colonization of mucosal surfaces. Overall, I found this manuscript to be interesting and thorough, combining several lines of evidence to understand mucosal colonization.
The investigators did a thorough and thoughtful job addressing my comments, and in my opinion, addressing the comments of the other reviewers.
Version 2:

Decision Letter:

27th September 2024

Dear Alex,

I am delighted to accept your Article "Pseudomonas aeruginosa faces a fitness trade-off between mucosal colonization and antibiotic tolerance during airway infections" 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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Due to the importance of these deadlines, we ask you please us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

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With kind regards,

[redacted]

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Reply to final comments from reviewers

Manuscript title: *Pseudomonas aeruginosa* faces a fitness trade-off between mucosal colonization and antibiotic tolerance during airway infections

Reviewer #2 (Remarks to the Author):

I would like to thank the authors for their thorough and detailed response to the concerns raised. I greatly appreciate that the authors have now included a variety of additional control experiments that further validate and support their findings regarding biofilm tolerance (e.g. cfu counts, MICs etc.). My points have been answered in detail, both in the rebuttal letter and in the newly added or revised text passages/figures in the manuscript. In my opinion, the adjustments contribute significantly to clarity and transparency and make it easy for the reader to follow.

All in all, this study is of great importance and relevance to the field, and I congratulate the authors to this excellent work!

> We'd like to sincerely thank the reviewer for recognizing our efforts on addressing the comments.

I have only a few very minor comments/suggestions:

Line 87ff: Grammar. "For example, AirGels are tissue-engineered 3D human lung organoids that replicate biophysical features of the airway environment, and that are suitable for high-resolution live microscopy"

> We modified the text accordingly (see line 92-94).

Line 105: It could be an idea to mention here that this work is based on PAO1 (since the Pseudomonas aeruginosa community mainly uses either PAO1 or PA14)

> We added the information about the strain used (see lines 107 and 110).

Line 604: Typo. "throughput" (instead of throughout)

> We corrected the typo (see line 479).

Line 746: Reference to Table S5 is not correct here.

> Corrected (see line 622).

Lines 903, 978, 980…: Typo. "gentamicin" (instead of gentamycin)

> Thank you! We corrected all these typos (see lines 421, 780, 856, 858, 881, 883).

Reply to the reviewer's comments

Manuscript title: *Pseudomonas aeruginosa* faces a fitness trade-off between mucosal colonization and antibiotic tolerance during infections

Reviewer #1 (Remarks to the Author):

Overall comments

- This paper is impressive, with a wide range of techniques used to provide multiple, independent layers of evidence, along with appropriate statistical tests and visualisation to demonstrate the key findings. A good read.
- Supplemental files provide good additional information.
- There could be more clarity surrounding the metabolic modelling aspects of the paper, though it does not hinge on these data.

We'd like to sincerely thank the reviewer for recognizing the value of our study and our experimental work effort.

Introduction

L39-40: Awkward phrasing of "we still know little...". Consider revising to "little is known about..."

We modified the text to address this point (see line 39-41)

L59-62: Nice. Good justification

Thank you!

Results

L114: I would remove 'very'. 0.7 = strong correlation, don't need adverb. Would also add "(r = 0.74)" to this line

We agree and implemented this change (see lines 124-125)

L127-129: Did the single gene knockout analysis for metabolic modelling also indicate this, when either lactate/glucose used as a sole carbon source?

Yes, this can be seen in our Table S4. When only lactate is provided as carbon source, *lldP* deletion prevents growth (fitness = 0). When only glucose is provided, *edd* deletion significantly decreases growth (fitness = 0.65). We now clarify this point in the main text (see lines 155-156).

L148-149: Were ornithine, proline, and arginine present in the simulated SCFM? If so, you could also test this by removing these from the SCFM media?

Thank you for this comment. Indeed ornithine, proline and arginine are present in the simulated SCFM as these were described in the original medium recipe (Palmer et al. 2007, *J. Bacteriol.*). We have now followed the reviewer's suggestion and performed

additional simulation experiments: (i) SCFM without ornithine, proline, and arginine; and (iii) SCFM where only ornithine, proline, or arginine are the only amino acids provided. These results can be found in the new Fig. 2C-D. We now also provide the recipes for all the media tested in our simulation in Table S5. Interestingly, we see that SCFM containing only ornithine, proline, and arginine now also group very close to our data, while SCFM containing all but these amino acids still group far away (Fig. 2D). This supports that these amino acids (in addition to glucose and lactate, which are also present in SCFM) are the important mediators of growth.

Linked to this - Fig 2C: I am wondering why the SCFM is so considerably different to the CF results in Fig 2C. What is the authors explanation for this discrepancy, which is essentially a lot of false positives in the SCFM modelling here. Does this indicate that the modelling is not all that accurate for these growth conditions?

HBE cells appear to only provide *P. aeruginosa* with ornithine, proline, and arginine as amino acids. Consequently, the wide variety of AAs in the SCFM medium leads to considerably distinct metabolic state. SCFM is however designed to model sputum of CF patients, not the mucosal surface. Due to its long residence time in the respiratory tract, we believe sputum composition differ in many ways from fresh mucus found near the epithelium. Sputum contains large amounts of dead cells, particularly neutrophils (a potential abundant source of AAs). Instead, HBE cultures and AirGels mimics the mucosal environment encountered by *P. aeruginosa* near the epithelial tissue. We believe the nutrient availability to be more similar to the ones found at earlier stages of infection (with mucins being the mostly available nutrient). We now further explain this point in the discussion (see paragraph in lines 345-362).

Fig 2D: It is not clear to me what the t-SNE is actually comparing here. Is it the result of growth under various media conditions for each single gene knockouts? This information is also absent in the methods section and could do with clarification.

We thank the reviewer for raising this point are sorry for the confusion. We confirm the t-SNE summarizes the signatures of fitness computed for individual single-gene knockouts for various media conditions. Each dot represents one medium for which the we simulated fitness (i.e. growth) as a result of disrupting each one of the 1,149 genes in the metabolic network, which we then compare to experimental data. To clarify this point, we extended the methods section (see lines 928-974) and added a new supplementary figure with a schematic representation of the approach (see new Fig. S3). In addition, the input table containing fitness values for each gene disruption/media is provided in Table S4.

Also, could you explain how this ‘circle’ was drawn? It seems arbitrary - was there any specific thresholds or clustering algorithms used? I don’t disagree with your general findings, as Orn, Pro and Arg are closer to your Healthy and CF points, but a systematic explanation/analysis is preferable here.

We agree with the reviewer that this was not especially clear. We arbitrarily drew the circle simply to highlight the conditions that were closest to our experimental data conditions in the t-SNE. In the figure derived for our updated analyses, we have now

removed the circle but further highlighted the difference between experimental and simulated data (see Fig. 2D).

Also, is there any MS data published which shows Orn, Pro and Arg being prevalent in the mucosa specifically, aside from “dead host cells, which can constitute a pool of amino acids”? This would be a useful reference if it exists.

We thank the reviewer for asking. To our knowledge, there is no measurement of the availability of specific amino acids at the lung mucosal surface (only in sputum). We hypothesize *P. aeruginosa* cells could be acquiring these from polyamines secreted by the host cells, which are thought be available during infections. An alternative hypothesis would be that such polyamines are secreted by some subset of cells within the library, feeding such amino acids for the remaining population. However, we think these 2nd hypothesis is much less likely given that arginine pathways have been shown to be essential under other infection contexts, such as urine or serum (Poulsen et al. 2019 - *PNAS*). We have now added some comments and references about this in our discussion session (see lines 352-356).

L155-157: To clarify, clustered based on what? What output was being measured here and clustered? I don’t necessarily disagree with the summary in 158-159, but this needs further clarification.

As stated above, we provide further clarification on the analysis pipeline. We are measuring patterns of fitness signatures under distinct simulated media compositions and comparing that to the patterns in our experimental data. Our new methods section should also make this much clearer (see lines 928-974).

L241: Do you mean 5A?. L249: Do you mean 5B?

Yes. Thanks for finding these typos. They are now fixed.

L267-269: Agreed. Very nice experiments here. Do you have a no antibiotic control?

Thank you for kind comment. Our controls for growth on the absence of antibiotics (Figs. 3 and 4 using Δ*wspF*; Figs. S6 using Δ*bifA*) and the new CFU data shown in Fig. S9 (where both strains were measured before and after antibiotic treatment) provide controls for this claim on the biofilm tradeoff. Regarding this specific experiment, we did not have a condition “no antibiotic” condition because the throughput of AirGels is still limited, and our goal here was to capture the tolerance heterogeneity.

L286-296: Really nice work with this, which shows the effect very clearly. Not sure if authors have considered this, but do planktonic cells provide any benefits to biofilm-forming cells? Or is it more of a commensalistic or even parasitic relationship? I am thinking of your point about the biofilms in L232 regarding their impact in: “maintain[ing] local host tissue integrity”. The depletion of epithelial cells in large biofilm (6C, last panel of top row) shows considerable depletion of epithelial cells. Is this actually negative for the biofilm-forming cells and thus, deleterious to the overall community? Not recommending any experiments or anything here. But say, comparing these results to S3 B at t = 10 h, I wonder if the impact on host epithelial cells and thus attachment for biofilm

causes an overall loss in biofilm? Maybe it's as you say though - "phenotypic heterogeneity and flexibility".

> In Fig. 6C, the epithelial cell depletion is mostly due to large Δ*wspF* biofilms pushing the cells and deforming the hydrogel. However, your suggestion about a "parasitic" relationship is interesting. The key would be heterogeneity within the population of acute-like cells. In a "parasitic" context, the majority of the acute-like population may deprive the nutrients from the mucosal surface (given their better spread/growth) while a minority still benefit from sheltering within the high-dense biofilms of high cdGMP mutants. This would be win/win for the acute-like strains when competing with chronic-like ones. However, an alternative scenario is also plausible: massive tissue damage by acute-like cells could provide nutrients to starving biofilm cells. In this case, the relationship would be more mutualistic. Because this is all speculation at this point, we preferred not to include a discussion about it in the main text. However, these are all exciting hypotheses to explore in the future and we think our models provide a physiologically relevant platform for further investigations.

Discussion

Methods

Methods are robust in general but more detail for computational methods is required

L629-630: What version of TPP and BWA MEM?

> We have now added the additional information. We used the TPP that came together with the TRANSIT v.3.2.7 and BWA v. 0.7.17). See lines 759-761.

L669-671: So if there was hypothetical/genes of unknown function, were they just not functionally classified, or were they were left out of additional analysis? If so, that doesn't seem right to me - highlighting these could lead to additional focused studies in the future where their function could be determined. A lot of the highest and lowest logfold change genes in the supplementary tables are hypothetical genes. I'd recommend adding an 'Unknown function' bar to 1C. It's important to highlight how much knowledge we are lacking.

> Thank you for this suggesting and we agree it is a fair point. Following reviewer 2 comments, we have now done a new enrichment analysis using Gene Ontology terms and KEGG pathways, which are more informative than the PseudoCAP classification. This new data is displayed in Fig. 1B, Fig. S2, and Table S2.

L684-685: What versions, what commands?

> We added this information (see lines 824-828).

For Transwells, what volume was added to well and to Transwell itself? Just the standard 600 µL into the well and 100 µL into the Transwell?

> We use 500 µL to the bottom side and 100 µL to the top side while culturing HBE in Transwells. We added this information in the methods (see lines 637-638).

L776-777: How? What tool/commands were run? If pyTFA, what version?

For the thermodynamic curation of the model, we estimated the Gibbs free energy of formation for the compounds and the corresponding error using the group contribution method (Jankowski et. al. 2008 – *Biophys* - PMID: 18645197), following the procedure described by Masid et al. 2020 (*Nat Commun* - PMID: 32499584). This information was translated into constraints in the model using the matTFA toolbox (Salvy et. al. 2018 – Bioinformatics - PMID:30561545), and more specifically the functions *prepModelforTFA.m* and *convToTFA.m*. The thermodynamic properties for the compartments of *P. aeruginosa* cells, including, pH, ionic strength, membrane potentials, and generic compartment concentration ranges were adopted from (Soh & Hatzimanikatis 2010 – *Curr Opin Micriobiol* - PMID: 25178783). We included all this information now in the methods (see lines 928-940).

L779-781: “We used the thermodynamically curated to assess…” should be “We used the thermodynamically curated model to assess…”? So do you mean that 40% of the encoded reactions in the PAO1 model were not used in analysis of growth in different conditions? If so, this may be an oversight by not considering genes from a well validated model. This may partially explain the differences seen in the simulated CF vs real CF results.

We are sorry for the confusion. No reactions were excluded from the model. This means that for 60% of the reactions in the model we have also thermodynamic constrains on top of the mass balance constraints. We tried to clarify this in the text (see lines 941-943)

L781-786: Could the authors please also provide the ingredient list for their simulated growth medias in Supp, Figshare or something equivalent? Ie what constitutes the ‘minimal media’? Also, what packages and commands were used to perform these simulations? Ie cobrapy for single gene knockout?

Thank you for the suggestion. As discussed above, we are now providing the ingredient list for all the simulated growth media (see Table S5). Regarding the packages, all simulations were performed in MATLAB 2017a and IBMILOG Cplex 12.7.1 as a solver. The *in silico* single gene deletion experiments were performed using the matTFA toolbox (Salvy et. al. 2018 – *Bioinformatics* - PMID:30561545), and more specifically the function *thermoSingleGeneDeletion.m.* We have now added this information to the methods (see lines 929-940,973-974).

L802: What was the input data for t-SNE?

The input file is the Table S4. We have clarified this in the Methods (see lines 966-969).

Reviewer #2 (Remarks to the Author):

In this manuscript, Meirelles et al. present a very interesting study based on a massively parallel sequencing approach of a *Pseudomonas aeruginosa* Tn mutant library pool grown on the mucosal surface of human primary bronchial epithelial (HBE) cells in ALI cultures and in an advanced organoid model (AirGels). This study implicates that biofilm formation is a fitness burden for bacteria during mucosal colonization (no additional selective pressure), whereas this burden changes into a fitness advantage under antibiotic stress. Furthermore, the authors show that hyper-biofilm formation not only confers antibiotic tolerance to the mutant cells themselves, but also cross-protects WT-like single cells in close proximity.

The combination of HBEs from healthy and CF donors in two different culture models (ALI and AirGel organoids) together with a PAO1 Tn mutant library is a novel approach to identify determinants of bacterial fitness on the established mucus surface (up to 3 months). Surprisingly, typical biofilm determinants, which are a hallmark of bacterial adaptation to chronic and persistent infections, showed a reduced fitness. The authors suggest that local nutrient limitation (due to limited exploratory behavior) reduces fitness of hyper-biofilm formers, but confers a selective advantage when bacteria are challenged with antibiotics.

I would like to emphasize and acknowledge that the authors have carried out a whole series of very lengthy and complex experiments to gain a more comprehensive understanding of *P. aeruginosa* adaptation during infection.

> We thank the reviewer for the very thorough read of our manuscript and for recognizing the complexity and value of the experiments we performed.

Major comments:

1) My main question is: What are the mechanisms behind the observed increased tolerance and the cross-protection of planktonic WT cells co-colonized with large aggregates of the hyper-biofilm former Δ*wspF*?

It is certainly a very interesting observation that biofilm producers only have an advantage under selective antibiotic stress conditions. Nevertheless, there are countless reasons that could lead to this tolerance (reduced penetration of the antibiotic, gradient of nutrients and oxygen etc.). Do you have a hypothesis?

> Thank you for this very good question. We hypothesize that nutrient and oxygen gradients generated within large biofilms reduces growth thereby causing an increase in their tolerance to CIP at the mucosal surface. Our data supports this hypothesis. First, we measured that the tolerance of the Δ*wspF* mutant correlates with the size of the biofilm (i.e. small biofilms are still susceptible, while large biofilms are tolerant). Second, the Tn-seq data shows a clear negative correlation between growth and tolerance for CIP. Furthermore, evidence from other studies show that CIP tolerance is linked to metabolism-related growth changes within biofilms. Finally, the effect is most likely not due to poor drug penetration as CIP diffuses well within biofilms and even tissues (Walters et al. 2003 – *Antimicrob. Agents Chemother.*; Stewart et al. 2019 – *J Bacteriol*).

Conversely, TOB has limited efficacy against biofilms due to a decrease in permeability (Tseng et al. 2013 - *Environ. Microbiol.*), which could explain the increased tolerance found by Tn-seq. Thus the mechanisms of tolerance at the mucosal surface are drug -dependent. We clarified our hypotheses and further discuss these mechanisms in the discussion (see lines 381-386).

2) In the first part of the study, the authors use a HBE-ALI model, while in the course of the study they switch to a complex organoid model (AirGel). For some experiments healthy donor cells were used, for others cells from CF donors. It is a bit hard to follow and I would appreciate if the authors would make it clearer which model and cell line was used for a specific experiment (and why).

Thank you for this suggestion. Cells from a healthy donor were only used for the 1st Tn-seq where we compared the results with Tn-seq in transwells from a CF donor. Because we saw no difference and because cells from our CF donor grow better, we subsequently only used CF cells. We have added sentences making it this clearer in the methods (see lines 622-625) and also in all figure captions.

Please include the specific advantages of the AirGel vs. ALI cultures and point out similarities/differences. Are the models interchangeable? What differences could you find in the Tn-seq output pools between ALI and AirGels?

The main difference is that transwells are higher throughput, while AirGels allow long time-lapse imaging. Therefore, transwells are ideal for bulk measurements (such as Tn-seq, see below) or end-point imaging, while AirGels are ideal for investigating growth dynamics (Fig. 2E-F and Fig S7) and response to antibiotics (Fig. 5D-F; Fig 6). The use of AirGels for measuring antibiotic tolerance is key because it allow the visualization of heterogenous survival to drug treatment by monitoring the exact same spot over long times (> 10h) with high quality imaging, something that is currently not possible with transwells. We have made this clearer in the text (see lines 81-89 in the introduction and 603-617 in the methods).

We performed Tn-seq in transwells for technical reasons: we need a large inoculum in order to avoid a strong bottleneck. Transwells are larger than AirGels (6.5 mm in diameter for Trasnwells membrane vs ~1.6 mm for the AirGel tube with ~4mm in length), allowing us to screen a larger inoculum. However, given that both models contain the same cell types and display similar physiology (mucus production and clearance), we would not expect much differences in the results, which is supported by our validations in both models (both in the presence and absence of antibiotics; Figs. 3-5).

Also, for further clarification, I would suggest that the authors adjust their simplified representations in each figure so that it is obvious for the reader, which particular model and cell line was used. For example:

- Adding a schematic to Fig. 3
- Adjusting the schematic in Fig. 6 (AirGel; similar to Fig. S3)

Thank you for the suggestion. To clarify this point, we have now modified Figs. 3 (added the label “HBE cultures” to panel C) and 6 (added the AirGel schematic).

3) The experimental design of the Tn-seq approach seem to have some weaknesses:

- Tn libraries were grown for 14h into deep stationary phase before being administered to HBE cultures (Line 541 ff). This already selects for and at the same time against certain mutants. I wonder how many genes of the PAO1 genome were actually covered by the inoculum used?

  Thank you for the comment, this is a very good point. We pre-grew the library to standardize the infection progression (i.e., all mutants would be already "active" when infection started and not coming from the -80 C). To reduce the odds of selection before inoculation, we used a high starting OD for this culture (OD = 0.25). As a result, our original transposon library had 4848 genes where at least 20 transposon insertions were detected, and our inoculum sample had 4780 genes with at least 20 transposon insertions detected, indicating only very minor reduction in population diversity. We have now added comments on this aspect in the methods (see lines 668-672).

  Furthermore, to make all this more transparent and allow reproducibility, we are now providing our analyses files (raw and aBGC normalized wig files) together with the annotation table we used. This will allow researchers to load these files in TRANSIT themselves and explore the dataset as they wish (e.g. repeat our analyses; visualize distributions of reads within their genes of interest; etc).

- Was there a bias within the inoculum towards mutants that have a fitness advantage in nutrient- and oxygen-limited conditions (as encountered in deep stationary, but also in biofilm conditions)? How many genes are enriched/depleted in the inoculum pool compared to the original Tn library?

  This is a great question. Because had both the library and the inoculum sequenced, we went back to our data and ran two additional analyses (see lines 778-782). First, we ran a comparison between the Tn-library sample and the inoculum. Then, we ran a comparison between the inoculum and the LB control sample. We have updated our schematics on Fig. S1B to account for these extra "quality control" comparisons. We now provide the results in Table S1 together with the other Tn-seq comparisons.

  For the Tn-lib vs inoculum comparison, we see that a total of 151 genes passed our significance cutoff. Among these, only 30 showed fitness defects with log_2-fold change < -1, and only one gene showed log_2-fold change > +1. Interestingly, this gene disruption that showed increase in fitness was *wspF* (log_2-fold change of 1.46). This made us initially think that maybe *wspF* could be under positive selection in LB cultures. However, the inoculum vs LB control comparison showed this was not the case, as insertion in *wspF* showed no effect. Importantly, none of the other biofilm-related genes (e.g., *bifA*, *algR*, *psl* operon) showed up in either of these comparisons.

  These analyses indicate no significant bias towards biofilm mutants in our inoculum. Since now we provide all the analysis files, researchers will be able to repeat these analyses and also check the distributions of their particular gene(s) of interest within all of our sequenced samples.

- The exposure times to the antibiotics were kept very short, followed by a long outgrowth step of 7-12h (Line 573ff). Does the population composition of the output

pool reflect differences in fitness as a response to the respective antibiotic or rather differences in (re-) growth capacity? (e.g. due to shorter lag times, higher doubling times…)

This is another very fair point. The outgrowth step is necessary to clear out dead mutants from the sample before sequencing. Also, short exposure was necessary to limit killing to ~1-2 orders of magnitude and avoid strong bottlenecks. We agree the differences are likely due to both an increase in tolerance and an increase in re-growth speed after exposure to drug. We have now added a note on this point (see lines 267-269). However, follow up experiments with CIP in AirGels (in which exposure is very long, Figs. 5E-F and Fig. 6) and now also CFUs (see new Fig. S9, which measure bulk tolerance) show that while decrease in lag may have happened during the Tn-seq experiment, we were able to screen for determinants of tolerance.

- As far as I understood, this study was based on one replicate per condition. Independent replicates would potentially have helped to identify noise and address my above-mentioned concerns

  We confirm that functional Tn-seq runs were performed once per condition due to the significant sample preparation and sequencing costs. However, as we explain in the methods (lines 674-676; 706-709), each sample contained material pooled together from ten different transwells, thereby representing an average of technical replicates. The purpose of the Tn-seq is to provide leads that are each time validated using clean deletions in multiple biological replicates. In fact, all our subsequent experiments with CFUs and imaging validated the Tn-seq data, which gives us confidence that the results are reliable. We also now provide the entire sequencing dataset, including the analysis wig files, which will stimulate the community to follow up with additional genes of interest.

4) The focus of this study is on the polysaccharides Pel and Psl and their regulation through c-di-GMP signaling, which also involves the Wsp system. However, I am missing the third polysaccharide alginate. The overproduction of alginate leads to mucoidity and is a hallmark of Pa adaptation during chronic infections. In addition, alginate production is also regulated by c-di-GMP (e.g. https://doi.org/10.1111/j.1365-2958.2007.05817.x; https://doi.org/10.1128/spectrum.00675-22). In particular, algR regulates c-di-GMP levels and biofilm formation through the diguanylate cyclase mucR (e.g. https://doi.org/10.1093/nar/gkv747). Both, algR and the related gene mucA, were identified as hits in some Tn-seq conditions. Therefore, the existence of alginate should at least be recognized.

Thank you for raising this very interesting point. We performed new competition experiments between a mucoid merodiploid mutant of *mucA* with WT PAO1 (*mucA* being essential, it could not be removed with clean deletions - see Schofield et al. 2021 - *Mol Microbiolol*). This mutant overproduces alginate, similarly to clinical strains. We competed the mucoid mutant with WT in LB cultures and at the mucosal surface of HBE transwells cultures. Similar to Pel/Psl overproducers, the mucoid strain showed fitness disadvantage only at the mucosal surface. As suggested by the Tn-seq results, these fitness defects were milder than the Pel/Psl overproducers (such as the Δ*wspF*). No fitness defect could be detected in LB cultures. These results are now

shown in a new supplementary figure (see new Fig. S5) and discussed in the results section (see lines 189-192). We also now introduce alginate early in the manuscript (see lines 55-56)

Along these lines, there was a recurring pattern, namely that loss of motility (e.g. flg and pil genes) increased fitness in mucosal colonization, but decreased fitness under antibiotic treatment. Similar to e.g. hyper-biofilm formers, these strains have limited exploratory properties (but due to completely different mechanisms).

Indeed, loss of flagella or pili genes increase fitness during mucosal colonization and decrease it during antibiotic exposure. However, Tn-seq cannot accurately reveal motility phenotypes due to redundancy between flagella-mediated and pili-mediated motility. Our previous work (Rossy et al. 2023 - *Plos Biology*) suggests that pili-mediated twitching motility is important to navigate at the mucosal surface and interact with mucus. However, flagella-mediated swimming may be important for nutrient foraging. Interestingly, mutants with Tn insertion in *pilT*, which still have pili but cannot use it, showed consistent fitness decrease during mucosal colonization (see Table S2).

Regarding the antibiotic tolerance part, our current hypothesis is that pili surface sensing is linked to certain efflux activation via activation of the cAMP pathway (Geiger et al. 2024 – *J Bacteriol*). Therefore, disrupting pili-mediated mechano-sensing (by removing major pilins like *pilA* or by preventing pili retraction by disrupting *pilT*) would decrease efflux activity when cells are on a surface. However, this is only a hypothesis which we are currently exploring in mechanistic details. Due to the purely speculative aspects on this topic at this point, we preferred to avoid bringing it up in the manuscript. However, if the editor or the reviewer find it necessary, we could add an additional paragraph on the discussion raising these points.

5) I am lacking some additional control experiments and information:

Regarding antibiotic treatment: Are there differences in the MICs for CIP/TOB between e.g. Δ*bifA* or Δ*wspF* and PAO1 WT? This would untangle whether the observed increased survival is due to resistance or tolerance.

We thank the reviewer for raising this point. To address it, we measure MICs of WT, Δ*bifA*, and Δ*wspF* for both CIP and TOB (fluorescent and non-fluorescent strains) (see Table S7). We could not detect any difference in MICs in either drug between any of these mutants. This further suggests that the effects seen are due to increased tolerance mediated by how these strains grow on the mucosal surface. We have now added this to the results section (see lines 296-298).

Was the tolerance only confirmed by fluorescence measurements or also by CFU counts? (e.g. Fig. 5)

This is another great point. We had initially only performed fluorescence measurements as it allows us to monitor the exact same area and correlate the survival with regions containing biofilms. However, we recognize the importance of having CFU data on this part as well. To address that, we have now also performed tolerance assays measuring CFUs (see new Fig. S9). We see the same trends of

increased tolerance in strains that overproduce biofilms. We have now added this to the results section (see lines 294-296).

Were control experiments with PAO1 overexpressing pel and/or psl performed? This would further confirm that other cdG-regulated traits make only a minor contribution to the observed phenotypes.

We have now performed tolerance experiments with Δ*wspF*Δ*psl*Δ*pel* (see new Fig. S9). Our results indicate indeed that the effect is mostly due to the production of the matrix. We have also now measured cdGMP levels in the tested strains and confirmed that they follow what we expected from the literature (see new data in Fig. S4). We have now added this to the results section (see lines 294-296).

6) In general, all parts of the methods section feel very lengthy, somewhat unstructured, and many passages seem redundant. All in all, this makes it a bit difficult to find the relevant information. Please shorten wherever possible and consider moving parts to the supplements.

We followed the editor's recommendations on keeping all the methods in the main text. However, we tried our best to clarify the methods section. We understand some parts seem repetitive; however, these are complex experiments and our hope is to provide all the details for maximum reproducibility. Information on apparently repetitive points (e.g. OD used for infection; removal of excessive mucus or not; etc.) are key for each experiment reproducibility and that is the reason why we provide them all in detail.

Additional comments:

Abstract:

Line 25: Wording. Repetition of limited/limits.

Corrected. We have slightly edited the abstract now.

Line 27: specify which biophysical mechanisms you are referring to

We slighted edited to abstract. Since we expect these mechanisms to vary for different drugs, we left the details on it for later in the paper.

Line 28: I feel like the term "genotypic heterogeneity" is not entirely correct here. Different mutations can lead to the same phenotype (antibiotic tolerance in this case), meaning that a genotypically heterogeneous population can indeed be phenotypically homogeneous (tolerant) without fitness costs. To emphasize the fact that susceptible cells are protected, I would rather suggest to use a term like: "population survival", "phenotypic heterogeneity", etc.

Thank you for the suggestion. We adopted the suggested "phenotypic heterogeneity" term (see lines 29-30)

Introduction:

Line 47: Wording. Since the transition from acute to chronic (and back) can be a reversible, I would suggest to use something like “strains with an acute lifestyle”, “strains that have adopted an acute lifestyle”, etc. instead of “acute strains”. The same applies to the section starting at lines 276ff)

We have made the suggested changes (see lines 48 and 308-333).

Lines 52 ff: Although it plays a minor role in the type strain PAO1, the existence of alginate should at least be mentioned (see point 4).

We agree and we have now added information on alginate (see lines 55-56).

Line 54: Introduce the abbreviation cdGMP when mentioning cyclic-di-GMP for the first time

This is now fixed (see line 55).

Line 56: You might want to add the following reference “Evolution of Antibiotic Tolerance Shapes Resistance Development in Chronic Pseudomonas aeruginosa Infections” (https://doi.org/10.1128/mbio.03482-20)

Thank you for the suggestion, we have now added the reference (see line 59).

Line 74: Wording. “Mimic” instead of emulate (see also line 304)

We changed the word to “model”, which we think captures the same idea (see lines 77).

Line 76: Wording. “do not take into account” or “do not consider” instead of overlook

We have now changed the sentence using “do not model” (see lines 79).

Line 83: Wording. I personally think that the term “functional genomics” is a bit exaggerated for a Tn-seq approach, as the phenotypic outcome is only assessed at the population level (mutant pool) and not for each individual mutant. I would appreciate if the term "functional genomics" would be replaced by "high-throughput massively parallel sequencing approach" or something along those lines. The same applies to lines 90, 364, 239.

Thank you for the suggestion. We have now replaced “functional genomics” for the more direct “transposon-insertion sequencing” throughout the text (see lines 98; 337 and 396).

Line 83: Wording. Repetition of tracking/track

This has been corrected (see lines 91-94).

Results

Line 93: Move the reference so that it appears directly after Tn-seq

This has been corrected (see line 101).

Line 95: Wording. “determined” instead of considered?

We have changed the word to “calibrated” (see lines 103-104).

Line 99: Wording. “mutants that were selected” or “mutants with altered fitness” instead of selected mutants

We have changed the wording accordingly (see line 107).

Line 103: Please add how many of the 620 genes were enriched/depleted. Which log2FC threshold did you use to identify significantly enriched/depleted mutants?

This information has now been added (see lines 111-112). We did not limit any log_2FC and instead used the software output of adjusted *p*-value < 0.05. We think this is important because we have a limited generation number that we can grow the library (due to inoculum/material size), which prevents high log_2FC for the mucosal colonization part. Because the software is already quite conservative, we opted to provide all the genes the passed the statistical cutoff here. We explain this in the methods (see lines 794-798).

Line 112: add the significance threshold used (adj. p val and log2FC)

This information has now been added (see line 122).

Line 120: Wording. “thrives in” instead of thrive to

We have changed the wording accordingly (see line 130).

Line 123: Add that you look at both healthy and CF HBE cultures.

This information has now been added (see lines 133-134).

Line 130: Wording. “produce” instead of make

We have changed the wording accordingly (see line 140).

Line 153: Add reference for sputum composition / synthetic sputum medium

This information has now been added (see line 164). Also, following reviewer #1 suggestion**,** we now provide a supplementary table with the composition of all simulated media (see Table S5).

Line 156: Wording. clustered with our “experimental” data most closely

The sentence has been re-worded following reviewer’s #1 comments (see lines 167-170).

Line 161: Wording. “penalty” is in my opinion a bit too bold. Please rephrase (see also lines 268; 398).

We changed it to “burden” (see lines 175, 299-300, and 449). If the reviewer also does not approve this, we’d welcome additional suggestions.

Line 174: Wording. “sessile” instead of sedentary? (see also line 219)

We have changed the wording accordingly (see lines 238).

Line 178: Does the ratio of the two competing strains have an influence on the colonization pattern? Did you test for other ratios? I could imagine that they are not necessarily co-existing 1:1 in the natural environment.

> We used a ratio of 1:1 because it is more feasible for microscopy. Accurately detecting fitness effects when other ratios are used (particularly if initially depleting the strain that loses the competition) could be more challenging by imaging, as many more fields of view would need to be analyzed. Because it is hard to know their exact ratios in the natural environment, we would argue 1:1 is a fair (although arbitrary) starting point. However, in the Tn-seq experiment, ratios are very different for each strain present in the pool, and hyper-biofilm mutants were still in disadvantage. Therefore, we think hyper-biofilm mutants will lose in these competitions no matter the ratio used.

Line 185: Briefly mention why it is advantageous to switch to AirGels. What do this model provide that is not covered by the previously used HBE cultures? (see point 2)

> The information has been added to the introduction (see lines 81-89). The advantage here is the fact that AirGels allows for long-term time-lapse microscopy. We also mentioned we used AirGels for time-lapses this in that part of the text (205-206).

Line 187: To me, it looks like continuous slow growth of Δ*wspF* throughout the experiment with a rather constant low growth rate. Please provide numbers to indicate the two different growth phases of Δ*wspF* (or rephrase)

> We have not rephrased the sentence (see lines 206-207). We now only point out that, as seen in the movies, Δ*wspF* forms big clumps after 11 h of infection that cannot disperse, while WT keeps spreading around the tissue.

Line 198: Wording. “forced to stay” implies outer forces. It’s more correct to state that the ΔwspF mutant (as well as the ΔbifA mutant) just do not have the capability to explore and disseminate (high cdG reduces motility). Please rephrase.

> We have changed the wording accordingly (see lines 216-218).

Line 220: Please clarify whether you have used healthy or CF HBE (see point 2)

> This has now been clarified in the text (see line 240). As discussed before above, everything beyond the mucosal colonization Tn-seq was done exclusively in CF HBE cells.

Line 221: Wording. Although 11h of infection is quite something for a virulent strain like PAO1, I would not call it “long-term” infection

> Fixed (see line 240).

Line 231: Wording. Specify under which conditions growth is impacted: Overall, while biofilm formation negatively impacts *P. aeruginosa* growth “during mucosal colonization” by limiting access to nutrients…

> We have changed the text accordingly (see lines 251-253).

Line 237: Wording. The header might imply that there is some tolerance towards colonization. Maybe better: “Colonization vs. tolerance trade-off…” or “Trade-off between colonization and tolerance for biofilms during antimicrobial treatment”. This also applies to Line 443.

We have changed the header accordingly (see lines 258 and 496).

Lines 237ff: The text refers to Fig. 6x, although this section is about Fig. 5. Please correct the entire section.

Thank you. The whole section is now corrected.

Line 239: Please state whether you have used healthy or CF HBE

This has now been clarified in the text (see lines 260-261).

Line 241: Please include the antibiotic concentrations relative to the MIC (e.g. 10x MIC). This also applies to lines 263; 283.

This has now been added (see lines 262-264).

Line 248: Wording. Re-order sentence

We have changed the sentence accordingly (see lines 273-274).

Line 251: Wording. Have any clean mutants with fitness defects during mucosal colonization actually been tested for their (biofilm) tolerance to ciprofloxacin? If not, please rephrase, e.g. “Several mutants displaying fitness defects during mucosal colonization showed increased fitness during ciprofloxacin treatment”

We have changed the sentence accordingly (see lines 276-277).

Line 255: Wording. Tolerance of the mutants was not tested (see above)

We have changed the sentence accordingly (see lines 280-281).

Lines 255ff: Please provide a separate list or mark the 24 hits that were found for both TOB and CIP-treated biofilms in Table S4. Mention in the text that some of the selected cdG-related example genes (displayed in Fig. 5C) were significant in only one of the two treatments. Also, I have difficulties finding dipA in Table S4. Please check.

Thank you for this suggestion. The list is now provided in Table S6. The information on passing the cutoff in at least one condition was added to the figure legend. Finally, we had included *dipA* for comparison given that it had come up in the mucosal growth part and showed opposite trends (although not significant) in the antibiotic treatment. We have now removed it from the figure and researchers can make this association by looking at the wig files, if they would like.

Line 260: Wording. “To better assess” or “To make accessible” instead of expose

We have changed the sentence accordingly (see line 287).

Line 266: Reduced biomass could possibly be an indicator of killing. Was the killing additionally quantified using PI staining (expecting an increase in dead cells over time)? Or CFU counts?

> We are convinced these are CIP-mediated dying cells given their massive reduction in signal and elongated cell shape of dying cells, visible in our Movies S5 and S7. PI staining in this scenario is difficult because it stains dead host cells too, which results in a very high background. As discussed before, our new CFU results (Fig. S9) should also address this point.

Line 277: Wording. “genetic” diversification

> This has been fixed (see line 309).

Line 278/280: Wording. Better: “strains displaying characteristics of planktonic (acute) and biofilm (chronic) lifestyle” or “strains adapted to chronic infection”

> We have changed the sentence accordingly (see lines 310-311).

Line 280: Doesn't the definition of planktonic (free-living single cells) exclude the possibility of these cells being in a biofilm? Please specify or rephrase.

> We agree with this point. We have now changed our terminology to exploratory/virulent (acute) strains, instead of planktonic (see lines 310-311).

Line 282: Why have you used Δ*wspF* although it was not amongst the hits of the Tn-seq experiment (CIP treatment)? Have you checked if Δ*bifA* shows similar cross-protection of WT cells as Δ*wspF*? Also, it is surprising that Δ*wspF* cross-protects, but didn’t show up as a hit in the Tn-seq experiment.

> **R.** This is a great question. Δ*wspF* is absent from the Tn-seq under drug treatment because it had already disappeared during the initial mucosal colonization step. We have also added a note on this in legend of Fig. 5 (see lines 504-506). However, our hypothesis was centered on cdGMP levels and biofilm formation. We thus preferred to use Δ*wspF* in the co-infection assays because it displayed a more pronounced biofilm phenotype. We have not tried co-infections followed by antibiotic treatment with WT and Δ*bifA* co-infections. However, in our experiments with Δ*bifA* alone, it was clear that it shows an intermediate phenotype, where certain regions in the field of view would form robust biofilms (and by consequence are more tolerant) while others show a more exploratory behavior (less tolerant). This can be seen in Fig. 5E and Movie S5. We think the heterogeneity of Δ*bifA* behavior is very interesting and it is likely one of the reasons why it mutates so often in CF patients, allowing it to benefit from an intermediate phenotype that still protects but does not pay such a heavy price on growth. However, given its complexity, this heterogeneity makes it less trackable for the specific cross-protection we wanted to test. We did not include a discussion on this given again its speculatory nature, but we could add a sentence/paragraph on this if the editor/reviewer find it appropriate.

Line 287: I assume that “rapid killing” indicates that planktonic cells and small clusters were already dead 4h post CIP addition. It would be helpful to additionally show images

from T0 (when the drug was administered) to illustrate this rapid killing (or define in which time frame “rapid killing” happened)

> Thank you for this comment. Cells only start dying (at least visually) in our assays after 3.5-4h of treatment. This is because it takes time for CIP to diffuse from the basal side of the AirGels through the gel and act on the cells. We now point out what we mean by this rapid killing (see lines 319-321) and point that the treatment dynamics can be seen in our Movie S7 (see line 526).

Line 293: see above (line 280)

> This has been now corrected (see line 328)

Discussion

Line 305: What is the difference in “chemical composition” of AirGels and ALI? Why are AirGels better in mimicking the chemical composition of the in vivo situation? Please specify or rephrase.

> Their composition is very similar, with HBE cultures (ALI in transwells) showing higher throughout and AirGels allowing high-resolution time-lapse microscopy. We rephrased the sentence to accommodate that we mean *in vitro* microtissue models in general (see lines 339-340).

Line 335: Wording. “mutational hot spots” instead of evolution

> We have changed the wording accordingly (see line 374).

Line 352: Wording. “massively parallel sequencing” or “Tn-seq” instead of “omics-based methods” (only one technique was applied)

> We have changed the wording accordingly (see line 396).

Figures

Fig. 1

Panel 1C: Since your work focusses strongly on genes involved in biofilm formation and cdGMP signaling, I would suggest to perform a functional enrichment analysis based on GO terms. This would be much more meaningful and supportive than PseudoCAP functional categories, which are quite broad. Additionally, since functional groups are very variable in the number of genes they contain, the representation as counts may be misleading. Please display the enrichment as % or fold-change enrichment or include at least the number of genes within each category.

> Thank you for this suggestion. We have now performed a new enrichment analysis using the DAVID webserver that we think is much more informative. This analysis includes GO Terms and KEGG pathways, and the results can be found in new Fig. 1B, Fig. S2, and Table S2.

Panel 1D: I suggest to highlight mutants involved in biofilm production in a different color to display their location on the correlation plot

We have now highlighted some of the biofilm-related genes in the plot (see new Fig. 1C).

Fig. 2

Panel 2B: I would find it more accurate to show growth as number of generations/8 hours.

We have modified the figure accordingly (see Fig. 2B)

Line 380: add "…compared to the LB control". This also applies to line 400.

This has now been fixed (see lines 430-431, and 450-452).

Line 385: Wording. "the red square indicates simulated growth"

We have changed the wording accordingly (see lines 436-437).

Line 389: Are the conditions within the circle manually selected by eye or has a statistical approach been used? (e.g. 95% confidence intervals) Please specify.

Following to reviewer #1 comments, we have changed the figure and removed the highlighting circle.

Fig. 3

Panel 3C: An additional merged projection would be nice to display colocalization of WT and the competing strain

The merged projection has now been added (see Fig. 3C)

Line 404/412: unify 1 to 1 and 1:1

This is now fixed (see line 456).

Fig. 4

Panel 4B/G: Could a histogram be more informative than the shown cumulative distribution?

We prefer to use the cumulative distribution as it does not require arbitrary binning. However, since we will provide spreadsheets with full data for every plot in the figures, the reader now should be able to download and plot the data, if desired.

Line 426/427: integrate the cdGMP part from the sub header of panel D into the main header

This has now been added (see lines 472-473).

Fig. 5

Panel F: Why does the fluorescence start at 0 (when CIP is added) and peak after approx. 5h? (see also Fig. 6E)

As discussed in the item above, cells still grow for around ~4 hours before they start dying. This is due to diffusion time from the drug from the basal part through the gel, plus the time necessary for visualization of CIP-mediated cell death by microscopy

(i.e. growth, unfinished cell division, and then cell burst). We have now added an explanation about this in the text (see lines 511-513)

Some of the Δ*bifA* replicates seem to resemble the WT patterns quite closely. Is this due to differences in aggregate size? Or what could be the reasons?

Yes, the data is heterogenous, that's why we think is essential to show each replicate. We noticed that Δ*bifA* displays a heterogenous behavior, with regions forming more robust biofilms while others showing a more dispersed phenotype. As discussed above, this can be seen even within the same field of view, as shown in Movie S5, and we think that is linked to the tolerance phenotypes. We added this detail to the legend of the Movie S5.

Line 453: Clarify what was quantified here (living cells)

This is now fixed (see lines 511-512).

Fig. 6

Line 464: Wording. "created" hints towards an active process. To make this statement, it would be necessary to show the same area before treatment.

This is an active process we commonly observed in the Δ*wspF* mutant. It happens after the biofilm reaches a large volume (~100-200 µm) and it was discussed in the "Biofilms mechanically damage epithelia while constraining pathogenicity" section of the paper. Data showing this formation is in Fig. S7B and in Movie S2-S3.

Methods

Line 484: I assume CIP was further diluted in water?

Yes, the 1000x stock was prepared in 20 mM HCl, and this is further diluted in water/PBS for application during experiments. The final volume added during experiments does significantly change the pH of the medium. We added a note about this in the methods (see lines 544-546).

Line 573: How were the optimal antibiotic exposure times determined/selected? Why was the antibiotic exposure time longer for TOB than for CIP?

The exposure times were optimized in order to obtain around two logs of killing of the library. For obtaining such values, a slightly longer exposure to TOB was necessary. We added a sentence mentioning this (see lines 704-705).

Line 658: log2 fold change (instead of chain)

This is now fixed (see line 792).

Lines 687 ff: Consider moving the section about cultivation of the HBEs to the beginning (e.g. directly before you describe the Tn-seq experiment in lines 536 ff).

This is now fixed. We moved this section to below the new short section where we explain the advantages of Transwells and AirGels and mentioned which model was used to each experiment (see paragraphs starting in line 619).

Lines 757 ff: Shorten and mention only relevant modifications to the previous paragraph

We tried to make the paragraph clearer and also now included a description of the tolerance experiments with CFUs (see paragraphs starting in line 830). We hope the reviewer will understand that each one of these assays have particular details that are key for reproducibility, and that's why they were included.

Line 779: A noun is missing "thermodynamically curated …"

This is now fixed (see line 942).

Line 708: explain what PDMS stands for

This is now fixed (see line 642).

Line 815: Were the main cultures grown in presence or absence of gentamicin?

The main cultures used for preparing the infection inoculum did not contain gentamycin (see line 1004). Since these are stable chromosomal integrations (*Tn7* site), the antibiotic is not needed. We just prefer to use it during the initial overnight preparation as a precaution.

Line 854: How was the "small cell size" defined?

This was done based on the *P. aeruginosa* cell size and our camera resolution, which for our data was 30 pixels. We have now specified this value in the methods (see line 1043).

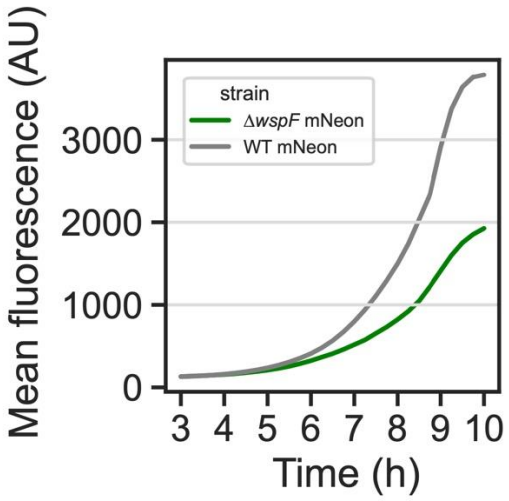
Lines 876 ff: Consider moving the section about imaging of bacteria directly before you describe competition experiments (lines 809 ff) and image analysis (lines 834 ff)

This is now fixed (see line 976).

Line 905: Are the two fluorophores used in this study comparable to each other in terms of stability and metabolic requirements to produce the fluorophore etc.? I am wondering if a control experiment was performed to prove that the observed differences in fluorescence were indeed due to altered growth and not due to biochemical differences of the fluorophores (e.g. by swapping the fluorophores).

Thank you for this comment. Our control experiments involving the competition of WT expressing mNeonGreen vs. WT expressing mScarlet did not show any difference (Fig. 3D), indicating there's no major differences in metabolic burden associated with making these two different proteins. Although we have not performed the exact swap experiment suggested by the reviewer, we had additional lines of evidence that the differences are indeed related to growth. First, we see exact the same trends when platting cells for CFUs, with magnified differences after 8 hours of infection. Second, we have also performed preliminary separate infections in AirGels (both strains expressing mNeonGreen), and the patterns are very similar (see data below). We have not included this data in the paper as it is preliminary, and the direct mean fluorescence is not comparable to the data in the paper (due to different exposure

time). However, it supports our point given that the trends are the same. Therefore, we think there is strong evidence that the differences displayed are related to growth effects.



Line 907: “Tamara’s”?

Thank you for catching this typo. The correct reference has now been added (see lines 1075).

Lines 952 ff: Which antibiotic concentrations were used? What are the MICs of the strains tested? (see point 5)

The concentration used (1 µg/mL) is mentioned in line 1129. The MICs of all the strains are now shown in Table S7 as discussed before.

Supplements

Fig. S1

Add relevant experimental details (e.g. about the bacterial inoculum [OD600; CFU; MOI…], duration of infection…) to panel B and/or the figure capture

This has now been fixed. See the updated caption of Fig S1. Additionally, all this information is available in the methods.

Fig. S3

A: AirGels were infected “with” WT or…

This has now been fixed (see the new legend of now Fig. S7)

Fig. S4

Wording, not all genes identified encode regulators. Maybe better: Tn-seq analysis identifies “genetic determinants” of antibiotic adaptation at the mucosal surface

Thank you for the suggestion. We have now modified the legend accordingly (see new legend of now Fig. S8).

Outgrowth for how long?

This information has now been added to the legend and is also described in the methods (see new legend of now Fig. S8).

Supplementary Table S1:

It would be helpful if you could add the functional categories to all genes and mark hits that you consider as being associated with biofilm formation/cdGMP signaling

This has now been done (see new Table S1).

What do Sum Ctrl, Sum Exo, Delta Mean represent? I cannot find an explanation

These were direct outputs from the TRANSIT software (number of detected reads and means for each insertion, etc). However, since now we provide the wig files and the analyses can be re-run), these are not very informative to readers. We preferred to keep the tables simplified in the new version of the manuscript, with only the log2-fold and adjusted p-value (see new Tables S1, S3 and S6).

Supplementary table S3:

Add *mexZ* to the respective PAO1 locus ID

This has now been done (see new Table S6).

General:

Unify healthy / non-CF HBE throughout the manuscript (e.g. Fig. 1 compared to Fig. 2/3)

This has now been fixed throughout the text.

Reviewer #3 (Remarks to the Author):

In this manuscript, the human airway mucosal surface was emulated leveraging tissue-engineered patient-derived airway organoids, which could mimic the mucociliary clearance function of the airway epithelium. With this organoid platform and the Tn-seq-based methods, the metabolic and dynamic mechanisms by which biofilm-dwelling P. aeruginosa experienced fitness trade-offs to adapt to the mucosal pressures and tolerate antibiotic treatment to initiate chronic infection were elucidated.

Overall, the study is well designed, clearly written, and represents a significant amount of work using very complimentary approaches and techniques. The authors presented interesting and important findings; however, I believe the following should be addressed before the manuscript can be accepted:

Thank you for kind comments.

Major comments:

1. Since airway organoids were used in this manuscript as a novel model to replicate the biophysical features of the airway environment, the typical features of the airway organoids should be clearly characterized to demonstrate its similarity to the actual airway epithelium, especially the cell composition and mucus secreting function. For example, to identify the ciliated columnar cells, secretory cells, etc. by fluorescent immunostainings.

   Thank you for this comment and we apologize for the lack of clarity. We have recently performed all the benchmarking and characterization of AirGels biology (see Rossy et al. 2023 - *Plos Biology*). In that paper, we characterized of ALI stability; histological signatures by single-cell RNA-seq and fluorescent immunostaining (basal, goblet and ciliated cells are present), and quantification of mucociliary clearance. We now make this clearer in the main text (e.g. see lines 86-89 in the introduction and 606-610 in the Methods). For clarity, we also provide the original figure from the paper below.

2. Expected as a bridge linking the gap between traditional “test tube” models and clinical studies, the organoid model facilitated with AirGels should be analyzed on its mucus composition by mass spectrum, especially for a comparison of the secreted mucus composition between the HBEs derived from CF vs. non-CF patients.

   We think this is an interesting suggestion. The characterization of AirGels shows that the mucus is predominantly composed of human Muc5AC and Muc5B, as it is found in the human lung (Rossy et al. 2023 - *Plos Biology*). While definite measurement of the glycosylation patterns of these gel-forming mucins are of great interest, detailed measurements by MS are non-trivial and pursued in subsequent studies. Tn-seq experiment was done in transwells, and we did not observe significant metabolic differences on genetic determinants important for *P. aeruginosa* to grow on the mucosal surface derived from cells of healthy or CF donors, further suggesting similar mucus composition in these *in vitro* models.

3. Two types of airway organoid models were applied, including HBE cultures on Transwells and AirGels. Please describe the similarities and differences between

these two models and explain the reasons for switching between them. Please convince the readers the advantages of AirGels over Transwells.

Thank you for this comment. This is something that came up in the comments from other reviewers too. As discussed above, we have now added a clear section in the introduction about the similarities and differences as well as their specific applicability (see lines 81-89). We have also added a section in the methods where we point out which experiments used each model (see lines 603-617). Important, it's not that one model is necessarily better than the other, they just serve different purposes. We developed AirGels to be able to study airway infection with high temporal resolution (time-lapses), which were key in our work to capture aspects related to virulence and tolerance of biofilm-producing strains.

4. The final step of AirGels chip fabrication was transition from immersed condition to ALI condition, in order to produce a lumen filled with air. Please provide evidences, such as cross-sectioned images of AirGels, to demonstrate the air-filled lumen and clearly show its dimensions, so that similarities between the model and the actual bronchioles can be visualized.

   Thank you again for this point. To clarify that, we're attaching below a figure from our previous paper where we developed the AirGel model. The air-filled lumen is visible by eye at the device (easy to distinguish from a filled lumen) and is displayed in panel C (right) below. The darker shades in the image are results of air-caused contrast in the lumen. If filled, the image would look like the one from day 0. All of our experiments with AirGels used AirGels with stable ALI inside the lumen. Regarding the AirGel dimensions, they are 1.6 mm in diameter by ~4 mm in length. We designed the model to match measurements of bronchioles (Chen et al. (2015) - *Respir Med*; Horsfield et al. (1968) - *J Appl Physiol*). We have now mentioned this in the text (see lines 648-649).

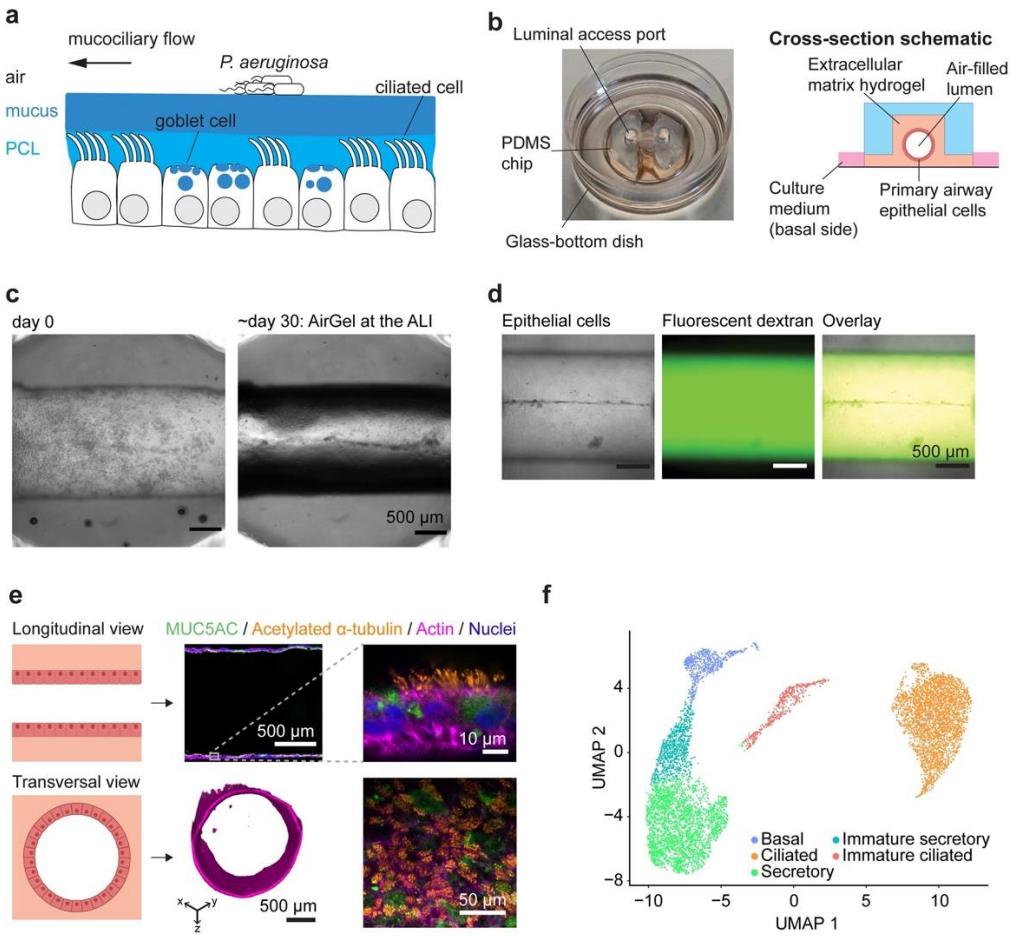


Fig 1. A tissue–engineered airway as a novel infection model.

(a) Simplified representation of the airway mucosa. Mucus–secreting goblet cells and beating ciliated cells are essential to generate mucociliarly clearance, which transports inhaled pathogens out of the airway. PCL, periciliary layer. (b) Picture and schematic of an AirGel chip. (c) Brightfield image of an AirGel on the day of HBE cell were seeded (left) and at the ALI after 30 days in culture (right). (d) AirGels permeability measurement by dextran assay. The brightfield image (left) shows the epithelial cells lining the lumen; the epifluorescence image (center) shows signal from the fluorescent 4 kDa dextran that does not cross the epithelial barrier, as shown in the overlay picture (right). (e) Longitudinal cross–sectional images of immunostained differentiated AirGels. Confocal images show the gel–forming mucin MUC5AC (green) and acetylated α–tubulin labeling cilia (orange) along with the actin dye phalloidin (pink) and nuclear dye DAPI (blue). The transverse cross section 3D image was reconstituted from SPIM data for actin fluorescence. The bottom right panel is a maximal intensity projection of a z–stack acquired in the curved lumen. (f) scRNA–seq identifies cell type diversity of AirGels. UMAP embedding of cells pooled from 3 differentiated AirGels, subjected to scRNA–seq profiling. The data underlying this figure can be found in S1 Data. ALI, air–liquid interface; HBE, human bronchial epithelial; scRNA–seq, single–cell RNA sequencing; SPIM, selective plane illumination microscope; UMAP, Uniform Manifold Approximation and Projection.

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This figure and legend can be found in Rossy et al. (2023) - *Plos Biology*

Minor comments:

1. Line 106-107: “Also, we found a surprisingly strong selection against mutants in genes coding for biofilm formation and cdGMP signaling (Fig. 1C, Table S1).”

Please list the terms in Fig.1C related to biofilm formation and cdGMP signaling.

Thank you. Reviewer #2 also brough this point. Therefore, we have now modified Fig. 1 to provide a more specific enrichment analysis using KEGG Pathways and GO Terms (see also Fig. S2 and new Table S2). We have also added notes on these genes in Table S1.

2. Line 115-117: “In particular, biofilm production was selected against in the CF HBE cultures despite their clinical dominance during chronic infections.” Please provide data supporting this conclusion.

We have now modified the correlation plot in Fig. 1 to highlight biofilm and cdGMP-related genes, so it’s clear what their fitness effects were (see new Fig. 1C). In addition, the entire section called “Biofilm formation imposes a fitness burden during mucosal colonization” explore this phenomenon (see paragraphs starting in line 175).

3. Line 248: “We found a mild negative correlation between fitness mucosal growth fitness.” Is the first “fitness” redundant?

Yes, thank you for catching this. We have corrected the sentence (see lines 273-274).

4. Fig1.D suggested CF and non-CF HBE cultures imposed similar selective pressures on P. aeruginosa. However, in clinical setting, CF patients much more frequently have extensive P. aeruginosa colonization in the respiratory tract. Please illustrate the possible reasons why there is no difference shown on the performance of P. aeruginosa in the current experiment settings employing CF vs. non-CF HBE cultures. Analysis on the mucus composition may shed more light on this issue.

Thank you for this comment. As argued above (see SCFM point of reviewer #1), our results indicate that CF HBE cultures are chemically similar to the ones from healthy HBE, at least for the two donors tested. We think these infection models provide a powerful tool for studying *P. aeruginosa* metabolism and growth at the mucosal surface, but at an early stage of infection. CF patients have a wide range of additional physiological characteristics that magnify their susceptibility to infection throughout their life. For example, CF patients develop think sputum that contain large amounts of host dead cells, which we do not capture in our models. In addition, given the sputum in the airway, their immune cells cannot combat infections properly, leading to more accumulation of dead cells (neutrophils) and disrupted pathogen clearance. Therefore, there are several aspects of CF biology that cannot be captured yet with *in vitro* models. We have added sentences on this in the discussion (see lines 348-362). Finally, the suggestion of analysis composition on mucus is an interesting one, but we believe it to be beyond the scope of our study. We think this would deserve its own story, where cells from multiple CF and healthy CF donors are evaluated to assess for specific variability.

5. R2 is a negative value in Fig5.B correlation growth vs TOB. Is it an adjusted R2? Please explain its statistical validity.

   Thank you for this comment. In this case, the coefficient of determination (R^2) was negative because the linear model does not fit at all the data. Additional information can be found here.

   This can already be seen by the distribution of the scatterplot and the very low Pearson correlation coefficient (0.06). We had simply added it to contrast with the much better fit found for the CIP data. However, we agree that it may be confusing and does not add any significance statistical validity. Therefore, we removed the linear fit for the TOB data and show instead only the Pearson correlation coefficient (r). See new Fig. 5B.

Reviewer #4 (Remarks to the Author):

The manuscript "*Pseudomonas aeruginosa* faces a fitness trade-off between mucosal colonization and antibiotic tolerance during infections" describes a TnSeq analysis of *P. aeruginosa* colonizing organoids that the investigators developed, and termed AirGels. The Airgels are "a tissue-engineered 3D human lung organoid model that replicates biophysical features of the airway environment and that is suitable for high resolution live microscopy". The investigators developed AirGels from epithelial cells and from human cells derived from cystic fibrosis patients. In TnSeq experiments, the AirGel organoids were colonized with a transposon library of *P. aeruginosa*, in order to identify genes with transposon insertions that caused reduced (or enhanced) fitness when cultured on the organoids, compared to other laboratory conditions. From the TnSeq analysis results, the investigators developed mutant strains with gene deletions, and performed imaging studies of the bacteria colonizing the AirGels, as well as competition studies, where the mutant strains were competed with wildtype to assess the sequence of events that occur during mucosal colonization by *P. aeruginosa*. They also used their TnSeq results to perform metabolic modeling and determine the nutritional requirements of *P. aeruginosa* when growing on the mucosal surface. Finally, the investigators performed a similar TnSeq experiment with *P. aeruginosa* cultured on AirGels and exposed to antibiotics tobramycin and ciprofloxacin. From their TnSeq results, the investigators focused primarily on genes involved in use of nutrients required for growth on mucosal surfaces and genes involved in biofilm formation, including polysaccharide biosynthetic genes and regulators of biofilm formation including genes involved in cellular cyclic-di GMP levels.

Overall, I found this paper to be novel and interesting in that it describes the lifestyle of *P. aeruginosa* as it colonizes human derived mucosal surfaces. The investigators describe genes important in mucosal colonization as well as genes involved in "exploratory" behaviors as the bacteria move to new nutrient sources on the mucosal surface.

> Thank you for the comments and positive feedback on our work. One note that we'd like to clarify to the reviewer is that the Tn-seq was not done in AirGels but in HBE cultures (transwells). Then, we used AirGels for time-lapse microscopy. Other reviewers had already suggested a clear description of these two models, which now we provide in the introduction, figure legends, and methods. We hope this will help readers clearly connect which model was used for each experiment.

My primary critique is that the text of the manuscript is often vague, and that the supplemental movies (while cool) are often difficult to interpret. For example: when the cells look like they are completely covering the surface, how is the reader to interpret the difference between the behavior of the mutant and the wild-type strain. For example:

Line 187: When competing with WT, ΔwspF displayed two successive phases of colonization (Fig. 3F). In an initial phase ΔwspF grew exponentially, yet with a lower rate than WT. Later (> 9 h), ΔwspF growth stalled, and WT grew dramatically faster. At this stage, ΔwspF formed large biofilms that could not efficiently disperse, whereas WT was able to colonize new environments (Movie S1).

We agree that this sentence was confusing, and we have now changed it (reviewer #2 also pointed this out). In addition, we have now added information to the legend of each movie, linking them to the specific data in the figures where it was quantified. Finally, we also added notes to the movie legends explaining exactly what we mean when we refer to the movies. We hope this will facilitate this interpretation.

I'm not really sure how to interpret Movie S1, to show that colonization occurred in two phases. A clearer description of how to interpret the data in the text of the manuscript would be useful. The same is true for the other figures. For example, please provide a better description of how cell death of both the mucosal cells, as well as the bacterial cells is interpreted. When describing the movies, it might be useful to include the statistics in the text, particularly when describing the differences between wild-type and mutant cells, as well as in the competition experiments. This would clarify the interpretation of the videos.

As described above, we followed the reviewer request and added new descriptions for all the movies. Now they contain the specific details needed for their interpretation added their legends (see Movies legends in the Supplementary materials). In addition, the part on growth phases was changed (see lines 206-207).

Minor points.

P. aeruginosa produces three different polysaccharides: Psl, Pel, and alginate. Alginate is not mentioned anywhere in the text. (although the strain used here, Pao1, is primarily a Psl producing strain). It can be induced to produce alginate or Pel, but it generally only produces Psl. CF Clinical isolates usually produce alginate.

Thank for this comment. Other reviewers had also pointed out the need to recognize the importance of alginate given its clinical relevance and the fact that we detected the *mucA* gene in our Tn-seq. We have now done so. See our discussion of alginate in the introduction (lines 55-56) as well as in the results (lines 189-192). In addition, we have now performed a competition assay between WT and a PAO1 mucoid strain. The results confirm that alginate overproduction also decreases fitness during colonization (see new Fig. S5).

The discussion section is short, and does not put the polysaccharide experiments in contexts of the literature (psl is not mentioned in the discussion. I think it could be expanded upon).

This is a great suggestion. We have now added a sentence on the costs of making Psl and alginate and their connection with nutrients available at the mucosal surface (see lines 350-355). In addition, we also connected the consequences of making these polysaccharides to drug tolerance (see lines 381-386).

A better description of *wspF* and *bifA* would be useful. When I looked up wspF, it is described on pseudomonas.com as "probably methylesterase". So, unless the reader is really familiar with the *Pseudomonas* literature, it is not clear how a methylesterase would be involved in the overexpression of ci-di-GMP.

This has now been fixed. We have now added a short description of the effects of mutating *wspF* (see lines 185-186) and *bifA* (see lines 202-203).