Oct 16th 2024

Point-by-point response to reviewers for antibiotics-3223074

Dear Editor,

thank you and the reviewers for taking the time to review this manuscript. We have addressed all the comments, and in doing so we have improved the readability of the manuscript. Below, you will find a point-by-point response (in blue) to the reviewers' comments (in black). Text highlighted in yellow indicates edits made in the manuscript, which are also visible in the track changes of the re-submitted file. Changes made in Figure 1 and Table 1 are signalled with a comment box next to these objects in the re-submitted file.

Three additional points are worth mentioning: 1) Following Karina Yang's suggestion, the manuscript has been reviewed for English language by an experienced English-speaking colleague (now named in the acknowledgments section). 2) In response to the editorial request, we have now acknowledged the use of OpenAI's ChatGPT-4, which was used exclusively for proofreading purposes. 3) Also addressing an editorial request, we have now updated the "Data Availability Statement" to include the accession reference for the raw whole-genome sequences deposited in the NCBI Sequence Read Archive.

On behalf of all the authors, we hope you find now the manuscript suitable for publication.

Sincerely,

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Reviewer 1's comments and suggestions for Authors:

1. The work is very interesting to the relevant sciences. However, massive improvement must be done in the text. In particular, i have found a lot of sentences started with We.

lines 95-131, etc. therefore, the whole text should be considered or revised.

Thank you for highlighting the relevance of our study. We have thoroughly revised the manuscript to address the writing concerns. The language has now been edited by postdoctoral researcher Dr. Jake Barber, a native English speaker with expertise in the subject matter (Google Scholar: Jake N. Barber), who is now acknowledged in the revised version of the manuscript.

2. Authors should make a Table and explain the fifteen mutants position (DNA sequence) of each mutants P. aeruginosa. explain how these mutations affects the strains roles in fosfomycin resistance mechanism in Pseudomonas entomophila.

Table 1 already shows the exact base changes and locations of the 15 mutants we identified. We have now added a column to indicate which domains of the GlpT protein are affected, highlighting that 12 out of 13 mutations occur in the transmembrane regions. These regions have strict sequence constraints, making mutations in them highly likely to be disruptive (Penn et al., 2020, *Sci Adv.* PMID: 32181350). In *P. entomophila's* GlpT, transmembrane domains comprise only 29.7% of the protein, yet 92.3% of the resistance mutations occurred in these domains. This deviation from the expected distribution is statistically significant (binomial test, *P-value > 0.001*), strongly suggesting that fosfomycin resistance in these mutants results from the disruption of GlpT's transport function. Further supporting this interpretation, 6 out of 10 unique mutations are frameshifts or premature stop codons, which by definition result in aberrant or truncated versions of the protein, respectively. This explanation has now been added to the text, including the Penn et al. reference.

3. Massive language edition is required is the whole text, it seems the texts are made using AI. please rewrite to make a clear flow of the study.

Done (see first answer).

Comments on the Quality of English Language:

Substantial level editing is required in the whole text. should be edited by a certified professional editor to make it sound

Done (see first answer).

Reviewer 2's comments and suggestions for Authors:

In this study, Sánchez-Maroto et al. worked to test a GlpT-based resistance mechanism in *Pseudomonas entomophila*. Although the study is well formulated, the following points need to be addressed.

Thank you for your positive apprasal of the formulation of our study and for taking the time to offer suggestions for improvement.

Points of major concerns:

1. The authors proposed that the 9 mutations were associated with alterations in the corresponding protein. How did they build this assumption? There are no experiments of the resultant proteins.

As we detailed in our response to Reviewer 1's second question, we have expanded on our interpretation of these mutations as being disruptive to the protein. For one thing, please note that 6 out of the 10 unique mutations cause frameshifts or premature stop codons, which will lead to aberrant or truncated versions of the protein, respectively. In addition, all types of mutations occur disproportionately in the transmembrane domains, whose functionality is known to be highly sensitive to mutations (90.9% observed versus 29.7% expected, P-value = 1.3×10^{-4}). This explanation has been incorporated into the text, and we have added a new column to Table 1 that details the GlpT protein domains affected by our identified mutations.

2. Figure 1 makes me confused. The authors reported that no *glpT* mutations were detected in the WT strain, however in the graph, there is still mutations within the WT bar!

Thank you for highlighting this potential source of confusion. As noted in the figure caption, the 'wt' bar indicates the absence of mutations, meaning the *glpT* sequence matches the wild-type. We have updated the y-axis label to 'glpT mutations' and changed the 'wt' label on the x-axis to 'none.' For consistency, these naming changes have also been applied in Table 1.

3. I am wondering, why did the authors looked only for a region of 100bp upstream of *glpT* during investigating the promoter, which could normally include 100-1000 bp?!

Bacterial promoters typically extend over 70 bp upstream from the transcription start site (PMID: 22156164). Based on the well-characterized *glpT* promoter region in *E. coli*, it is known that the major repressor, GlpR, binds to the region between -51 and -32 in the *glpTQ* promoter (PMID: 1556120). The primer we used (as detailed in section 4.3 of the paper) binds to a region 142–123 bp upstream of *glpT*, therefore effectively covering the important -70 bp region while allowing us to effectively use the reverse primer initially designed for amplification. We have added a brief clarification in section 4.3.

4. *P. entomophila* is known to express *fosA* which is regarded as fosfomycin resistant gene. It is surprising for me that authors did not investigate whether there is a connection between *pho* and/or *glp* and this gene.

You are correct that *P. entomophila* carries a *fosA* gene in its chromosome, although little is known about its precise function and expression levels. FosA homologs are commonly found in many Gram-negative bacteria and are believed to contribute to intrinsic fosfomycin resistance; that is, their deletion increases basal levels of sensitivity (PMID: 28851843). We were totally open to the possibility that fosfomycin resistance might be mediated by the overexpression or modification of *fosA,* or any other locus for that matter. However, the reality is that we found no evidence to support this possibility; WGS showed that the only two resistance mutants without mutations in *glpT* carried mutations in *phoU*.

Points of minor concerns:

1. I find it somehow strange, to use the 125 ug/ml of fosfomycin and justifying this by founding it in a study performed in *P. aeuroginosa*. The authors need to do Luria-Delbrück fluctuation assays using data based on studies performed in *P. entomophila*.

We appreciate your concern, but please note that *P. entomophila* is a relatively new species, first described in 2005, which actually highlights the novel contribution our work makes to the field. Given the limited research available on *P. entomophila*, we followed the conditions validated for the closest species with relevant studies, which is *P. aeruginosa*.

2. Please indicate in table 1 that the stars refer to the two strains with nonsense mutations which used later.

Thanks for pointing at this missing information. We have now included this information to the Table description.

3. The authors need to include a diagram indicating the component of *glpT* and *phoU* loci.

Thank you for your suggestion. However, as this manuscript is a short communication, we believe that adding an extra figure to present information that is publicly available in appropriate databases (such as the one referenced at https://mage.genoscope.cns.fr/) would be unnecessary and could divert the reader's attention.

4. In figure two, how many replicates were used to measure the MIC in graph 2, B?

We appreciate you pointing out this missing detail. We conducted the MIC measurements in graph 2B with three replicates, with no observed deviation among them. We have now included this information in the corresponding figure caption.

5. In line 294, the authored mentioned that the 0,5 McFarland is equal to more or less 1 x 10^8 CFU/ml in *E. coli*. They can not use this value for the *P. entomophila*. This will raise the question again what was the CFU values used in these experiments?

Thanks for raising this issue. You are right the value from *E. coli* should not be directly applicable to other species. We just used it as a conventional method for standardizing

cultures. Indeed, for *P. entomophila*, our measured value was notably lower, at *1.14 ± 0.4 × 10^7* CFU/mL (mean ± standard deviation, n=3). We have now amended the relevant section.

Comments on the Quality of English Language: The quality of writing is average and could be followed.

We are glad you were able to follow the text. At any rate, in response to Reviewer 1's first comment, the language has been edited by a postdoctoral researcher and native English speaker with relevant expertise in this area (now in Acknowledgements).

Reviewer 3's comments and suggestions for Authors:

The study investigates fosfomycin resistance in *Pseudomonas entomophila*, comparing it to *Pseudomonas aeruginosa*. The authors show resistance in *P. aeruginosa* is linked to mutations in the glycerol-3-phosphate transporter (GlpT), some *P. entomophila* mutants had mutations in the phoU gene, reducing glpT expression. The study is not very well planned, the experiments performed are not enough to conclude the said findings. Here are my comments:

1. 2.1. Fosfomycin resistance in *P. entomophila* involves mechanisms beyond glpT inactivating mutations: The authors studied these mechanisms on the spontaneous resistant mutants of Fosfomycin. This study need to be performed on the resistant mutants passaged multiple times and then passaged at least for 7 days in the drug free media to know if these are stable mutants. Then these types of conclusions can be derived.

Thank you for this suggestion. The mutants were re-streaked at least once under antibiotic selection pressure after isolation to ensure both purity and stability, following standard practice in microbial genetics (e.g., see recent examples in high-profile journals: PMID: 32130952, PMID: 38175839, PMID: 36934122). Passaging mutants in drug-free media for several days could be interesting, as it might reveal secondary mutations—either enhancing resistance or compensating for fitness costs. However, this would represent a separate line of investigation, unrelated to the conclusions of the present study.

2. The heading 2.1 and 2.2 are same.

Thank you for catching this oversight. The heading 2.2 has now been changed to "Mutations in *phoU* confer fosfomycin resistance in *P. entomophila* by reducing GlpT expression".

3. Figure 1B does not explains properly. The authors could use a different graph such as bar graph with the axis plotting the frequency of mutations.

Thank you for signaling this potential source of confusion. Figure 1B is indeed a bar plot, but it is combined with a representation of gene length to show mutations are scattered throughout the sequence (which conveys the relevant information that they are likely loss-of-function mutations). We have revised the figure caption to clarify this and to remind the actual values to the readers; that is, as shown in Table 1, all mutations occur only once, except for the deletion at codon 423, which appears five times.

4. Figure 2B, the Y-axis labels are missing, therefore it is very difficult to see the MIC values. Please label the axis properly.

Our version of the Figure 2B is properly labeled (plase note that it uses a logarithmic scale, where, by standard practice and convenience, only the major order of magnitude marks are labeled). Just in case, we would ensure with the editors that there was no issue with the PDF file conversion.

Comments on the Quality of English Language: The quality of english is moderate and can be improved.

As already stated in the response to Reviewer 1, the language has been edited by a postdoctoral researcher and native English speaker with relevant expertise in this area (now in Acknowledgements).

Reviewer 4's comments and suggestions for Authors:

Comments and Suggestions for Authors

Pseudomonas entomophila is a widespread bacterium capable of killing insects across 8 orders, making it a model for host-pathogen studies and a promising tool for biological pest control. Laura et al. identified 15 fosfomycin-resistant mutants, two of which had mutation in *phoU*, a regulator of the phosphate starvation (Pho) regulon. The data revealed an atypical Pho regulon control of *glpT*, suggesting that this regulation is likely specific to the habitat and lifestyle of *P. entomophila*, a configuration uncommon among other pseudomonads. This highlights how ecological differences can drive rapid divergence in resistance mechanisms, indicating that spontaneous antibiotic resistance may cause by diverse genetic elements.

Thank you for this accurate summary of our work and its implications.

I have several comments according to this manuscript: Laura et al. gave a novel idea that the same spontaneous antibiotic resistance can lead by different genetic changes. This is also excellent combination of experimental data and computational data. However, more solid experimental evidence should be provided to support this novel atypical regulation pathway, especially the computational analysis indicates that *phoB* can bind to *glpT* promoter.

Thank you for recognizing the novelty of our work and praising the value of its combination of experimental and computational approaches. Regarding your suggestion, we agree that while our experimental evidence provides a solid proof of concept linking the *pho* regulon and *glpT*, additional work is necessary to elucidate the precise molecular details. Working out such details in a non-model species will require substantial experimental load (mutant construction via CRISPR or classic allelic replacement, gel shift or chromatin immunoprecipitation assays) and it could easily take many months to complete. We believe that this line of investigation, while interesing, falls beyond the scope of a short communications paper, and we hope it will be pursued by our group and others in the near future. This need for future studies is now briefly acknowledged in the "Conclusions" section.

Majors:

1. In figure 1 panel B, author should map those mutations onto the GlpT structural model, this may make the figure more informative and easier for reader to understand where those mutations are.

Thank you for the suggestion. We considered mapping the mutations onto a 3D structural model of GlpT but found it not very informative for two main reasons: 1) GlpT is a multipass membrane protein with 12 α-helical transmembrane domains, making the 3D structure challenging to visualize in 2D (as would be in a static figure). 2) The mutations are scattered along the *glpT* sequence rather than clustering in a few functional regions (e.g., an active site). Actually, the only notable pattern is that most mutations occur in transmembrane domains (90.9% observed versus 29.7% expected, P-value = 1.3×10^{-4}; analysis now included in the text). Given these considerations, any realistic structural representation of the protein and its many mutants gives rise to a visually cluttered figure, not really very informative.

To efficiently convey the important structural information, we made the following adjustments: 1) We added a column to Table 1 indicating the specific domains affected by each mutation, highlighting that 13 out of 14 mutations are in transmembrane regions. 2) We now also discuss in the text that this significant bias of mutations toward transmembrane domains strongly suggests loss-of-function effects, as mutations in these regions are known to be highly disruptive (Penn et al., 2020, Sci Adv. PMID: 32181350). 3) To highlight the random distribution of mutations across the *glpT* sequence, we originally chose the linear representation shown in Figure 1B. However, since your comment made us realize this random configuration might be not obvious for some readers, we have now made this feature explicit along with its interpretation in the figure caption

2. In figure 2 panel A, author presented the *glpT* expression level among *phoU* mutant and wildtype. However, *phoB* is the transcription factor which directly interact with *glpT* promoter. Author should include *phoB* mutant or knockout samples into the *glpT* expression level experiments, especially author also found out that there is a *phoB* binding site within the *glpT* promoter sequence. This might help to confirm that GlpT is control by Pho regulon in *P. entomophila*.

Thank you for the suggestion. We agree that while Figure 2A establishes a clear experimental evidence linking the *pho* regulon to *glpT*, additional work is necessary to clarify the precise role of PhoB as a mediator. However, we currently lack well-defined expectations regarding *glpT* expression levels in a *phoB* knockout mutant. While PhoB typically acts as an activator of *pho* genes, it is also reported to function as a repressor of other genes (hence its classification as a dual transcriptional regulator in, for instance, RegulonDB, ref: RDBECOLITFC00202). If PhoB serves as a repressor of *glpT*, we might expect *glpT* expression levels to be either similar to or above wild-type levels in a *phoB* knockout. Conversely, if PhoB is an activator, *glpT* expression could be either similar to or below wild-type levels. So it is unclear whether the experiment you suggest would provide much usable information. Actually, resolving the actual role of PhoB in detail would require further experimental work beyond qPCR, such as mutant construction via CRISPR or classic allelic replacement, and techniques for elucidating physical interactions between protein and DNA like gel shift or chromatin immunoprecipitation assays. Given the time and complexity involved, particularly in a non-model species like *P. entomophila*, we believe this falls beyond the scope of a short communication. Instead, we feel that rapidly sharing the combination of experimental and computational evidence presented here will promote further research into this system. This need for future studies is now briefly acknowledged in the “Conclusions” section.

3. Figure 2 panel B should include the error bar and indicate the repeat times of the MIC experiment.

We appreciate you pointing out this missing detail. We conducted the MIC measurements in graph 2B with three replicates. Since all values were consistent for each strain across the replicates, adding error bars is unfeasible (and unnecesary). We have now included this information in the corresponding figure caption.

Minors:

1. The subtitle 2.1 and 2.2 are identical, please combine these two sections or give a new subtitle.

Thank you for catching this oversight. The heading 2.2 has now been changed to “Mutations in *phoU* confer fosfomycin resistance in *P. entomophila* by reducing GlpT expression”.

2. Figure 3 should indicate panel a and b corresponding to the figure legend.

Thank you for highlighting this potential source of confusion. Since this is effectively a single figure, we have modified the caption to explicitly indicate which elements we are referring to (e.g., 'the neighbor-joining phylogenetic tree on the left is based on…' and 'the heatmap on the right illustrates the abundance…').

3. In line 78, author mentioned that there is no functional *uhpT* homologue in *P. entomophila*, are there any *glpT* homologue exist in *P. entomophila*? If so, what is the protein sequence identity?

Yes, it is the gene we sequenced to produce Table 1. The percent protein identity is 83.3%. We have now included this information in the appropriate passage.

4. In the discussion, author should discuss the relationship between the atypical GlpT regulation and the living environment and lifestyle of *P. entomophila.* What makes *P. entomophila* adopts this special GlpT regulation pathway.

A whole paragraph dedicated to this can be found between lines 223 and 234. Briefly, we suggest that the abundance of glycerol-3-phosphate in insects (both in flight muscles and in larvae) could have provided the selective opportunity for the integration of GlpT under the Pho regulon, enabling an entomopathogen such as *P. entomophila* to utilize glycerol-3-phosphate as both a carbon and phosphate source.