nature portfolio

Peer Review File

Heterogeneity in the response to the metabolic environment drives fosfomycin heteroresistance

Corresponding Author: Dr David Weiss

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Work by Choby et al. explores the mechanism by which Enterobacter cloacae complex heteroresistance leads to treatment failure. Through a Tn-seq analysis, the fosfomycin resistant determinant FosA was identified as a requirement for high-level fosfomycin resistance (growth at 256 µg/mL). Other mechanistic studies, including elegant promoter-reporter assays, revealed variation in the expression of the gene encoding the glycerol-3-phosphate importer GlpT across the population, likely resulting in heteroresistance. The presence of glucose also decreased glpT expression, proposing a potential relevance of this phenotype in diabetic patients. Interesting, testing a type 1 and 2 diabetes in vivo models showed increased Mu208 CFUs growing at 256 ug/mL fosfomycin. The report is well written and describes an interesting observation of potential clinical relevance; however, some of the main conclusions lack support from the data currently presented.

Major concerns:

1. Fig 1d-e: Cause of in vivo treatment failure. The link between HR and failure cannot be established based on the presented results. A good proportion of the population is growing at 256 (the breakpoint, Fig 1a) so it is not clear if failure is due to HR or just overall higher level of resistance compared to the Mu819 strain susceptible to <32 ug/mL. Notably, in figures 1d and 1e, both strains were treated with the same fosfomycin concentration, but Mu819 is already susceptible, while Mu208 is resistant. Similarly, Figure 1i wild-type is resistant, and Figure 1j ΔgshA is susceptible, raising similar concern that the data would suggest that the treatment failure is due to resistance, not heteroresistance. Strains that have comparable fosfomycin MIC but differ in whether they are HR or not should be tested.

2. Fig 1j & extended Fig 2c: Interesting results but they do not necessarily mean that endogenous production is absolutely required but may have to do with the level of GSH in the host. In vitro, exogenous GSH does not chemically complement the WT phenotype (Extended Fig 2c) although that was performed at a single GSH concentration. There is a potential that the in vivo level of GSH in mice (or that exogenously supplemented in vitro) is not high enough relative to normal endogenous production. The chemical complementation experiment should be done in dose and higher levels of in vitro supplementation should be tested. Also, a discussion on the relative abundance of this metabolite in mice (& other mammalian models) and humans will then provide insights into whether the same effects may be observed in human or not - similar potential discrepancies were previously observed for certain vitamins and metabolites...

3. Low frequency glpT null mutants: this small subpopulation described in Extended Fig 3d (first half of P. 8) appear to be enriched by the antibiotic, potentially accounting for – at least in part – for the observed HR. It is not clear why this mechanism was largely dismissed in the subsequent sections exploring the different glpT expression levels. Importantly, how this subpopulation is accounted for in the mCherry experiments as these experiments do not consider the potential impacts of a null glpT mutation within the bacterial population. Controls addressing this should be included. Is there a potential for a dual mechanism or is one more dominant?

4. HR vs resistance: The choice to use a single concentration for many assays, equivalent to CLSI breakpoint, (e.g., Fig 3d, 4a, c, d, and others including in vivo exp in Fig 5) does not capture subpopulation level frequencies and may just reflect MIC shifts (i.e., resistance). In general, heterogeneity cannot be as conclusive by testing at a single concentration only. More concentrations, including sub-breakpoint ones, should be tested. That includes adding a few representative dose-response

PAP-like assays for the created reporters.

5. mCherry promoter reporter assays limit of detection. While these reporter assays offer a powerful approach to determine cell level expression and phenotypic effects, the limit of detection of this assay seems high compared to the PAP analysis. For example, if the frequency of a resistant subpopulation in 1 in 100,000, the promoter reporter will not be able to capture this subpopulation with the current assay design, which only includes 700 - 1000 cells. This is evident in the discrepancy between Figure 3b ΔglpR and Figure 3c, where there is a subpopulation surviving at 128 µg/mL fosfomycin in Figure 3b, but in Figure 3c, there is no resistant population. Testing more concentrations, as mentioned above, may partly mitigate this concern and a discussion about the limitation of this assay should be included.

6. In vivo diabetes experiments. Fig 5: This is an interesting experiment. However, it should be complemented with testing the effect of fosfomycin on the therapeutic outcome (or survival) of these diabetic mice relative to control, especially that the CFU differences are within the same log.

7. Heteroresistance definition. The authors refer to prior literature that offer standard definitions of HR but adapt them through four criteria. Notably, this group places emphasis on clinical breakpoints. While this is understandable to infer clinical relevance to the phenotypes, there should be a discussion acknowledging that the specific breakpoint value may change over time (as occasionally done by CLSI and others). Also, the criteria should align with similar studies from the same group number (such as https://doi.org/10.1038/s41467-025-60828-9) by removing reference to 2X breakpoint in the second criterion related to Level of resistance.

Minor comments

a) The first section in the Results section does not describe the investigation of the mechanism of fosfomycin HR. Therefore, the first sentence in this section should be revised accordingly.
b) Page 8, line 1: the data presented thus far in the manuscript can perhaps “hypothesize” but not “support” the model yet. Please revise this sentence.
c) For Figure 2d, the figure displays 256 µg/mL of fosfomycin while the caption says 128 µg/mL.
d) Fig 2c: please show more representative images (in supp or extended data fig) of this (glpT) and the control esp. for panels with mCherry expressed.

Reviewer #2

(Remarks to the Author)
Reviewer’s comments:

This is an outstanding and comprehensive study that elucidates a novel mechanism of fosfomycin heteroresistance in Enterobacter cloacae. The authors convincingly demonstrate that heterogeneity in the expression of the fosfomycin importer GlpT, regulated by host metabolic cues (specifically glucose), tunes the frequency of the resistant subpopulation. The combination of this heterogeneity with the "resistance enhancer" FosA drives the heteroresistant phenotype. The translation of this finding into murine models of hyperglycemia/diabetes, showing a clinically relevant increase in the resistant subpopulation in vivo, is a significant strength and highlights the potential impact of host physiology on antibiotic treatment outcomes. The manuscript is well-written, the data are robust and support the conclusions. However, several critical issues must be addressed before the manuscript is suitable for publication.

Major issues:

1.The paper introduces the paradigm-shifting concept that the host's metabolic environment (glucose levels) can directly modulate the frequency of an antibiotic-resistant Enterobacter cloacae subpopulation (fosfomycin resistant) during infection and author suggested that the metabolic context of infection in specific disease states should be considered when prescribing antibiotics. What I'm curious about is whether other bacteria such as the E.coli, Klebsiella, or Staphylococcusalso have a similar mechanism.

2.“ we observed that the resistant subpopulation was more frequent during infection in murine models of hyperglycemia/diabetes”. How about the clinical -derived beacterial that isolated from the diabetic, or other patients. It is suggested to conduct more research on the heterogeneity of resistance of clinical strains to fosfomycin. Whether the mechanisms of heterogeneity resistance of these clinical strains to fosfomycin are all universal.

3.Mu208 is the Enterobacter kobei clinical isolate while MU819 is Enterobacter cloacae clinical isolate. What are the differences between these two types of bacteria? Will this affect their antimicrobial phenotypes?

4.Enterobacter cloacae strain and treated them with antibiotics to investigate the possible treatment failure caused by heterogeneous strains. However, we know that Enterobacter cloacae is usually a conditionally pathogenic bacterium and generally does not cause disease in normal mice. Did the author evaluate the pathogenicity of the mice?
5.“Mu208 exhibited fosfomycin HR, harboring a subpopulation of 1-10% of cells resistant to 256 µg/mL fosfomycin and greate” It best known that when antibiotics are used, they can kill the sensitive bacteria while increasing the number of drug-resistant bacteria. How was this data calculated?

6.“Therefore, the fosfomycin resistant subpopulation requires FosA and its cofactor glutathione” ,“ To test whether bacterial-derived glutathione is required even in the presence of mammalian-derived glutathione, we infected mice with a lethal dose of the ΔgshA glutathione synthase mutant, and found that this strain did not cause treatment failure, Thus, synthesis of the FosA cofactor glutathione is critical to the fosfomycin resistant subpopulation and the ability of strain Mu208 to cause fosfomycin treatment failure in vivo”. I’d like to know the results of the overexpression strain of gshA.

7“glpT inactivation confers fosfomycin resistance”and “Thus, there is a rare subpopulation of resistant cells with null glpT function, but the majority of the resistant subpopulation has intact GlpT” . Here is really confusion.

8.“Expression of the fosfomycin importer GlpT is heterogeneous and predicts survival in Fosfomycin”. We know that the MIC values of different resistant subpopulation Are different. Are the expression levels of GlpT in these different subpopulation consistent? How about different clinical strains.

9“Transcriptional regulators GlpR and CRP control glpT expression and fosfomycin resistance”reveals the key regulatory axis of "glucose-CRP-glpT", providing an important mechanism for understanding how carbon sources affect antibiotic resistance. However, it has limitations in terms of physiological relevance, the complexity of the mechanism, the completeness of technical evidence, and the precise definition of resistant subpopulations. It is suggested that gel shift assays (EMSA) or chromatin immunoprecipitation (ChIP) could be used to verify the direct binding of CRP protein to the glpT promoter.

10.“We measured glpT transcript abundance by qRT-PCR and found that excess glucose resulted in ~100-fold reduction in relative glpT transcript abundance (Figure 4b), confirming that glucose increases fosfomycin resistance via glpT repression.”Even though the expression level of glpT increased in the presence of glucose, this might be the main reason for the increase in drug resistance. Are there any other possible causes?

11.“When Mu208 was grown with glycerol as the sole carbon source, the frequency of the subpopulation resistant to 256 μg/mL fosfomycin was below 1%, but low concentrations of exogenous glucose resulted in a dose-dependent increase in the frequency of the resistant subpopulation (Figure 4c). Additionally, the ability of glucose to enhance the resistant subpopulation appeared to be common among 5 fosfomycin HR Enterobacter clinical isolates (Extended Data Figure 6). ”Only under the condition of glycerol as the carbon source, the nutrients required for bacterial production are relatively less. Is this phenomenon caused by this reason? Figure 4 showed that as the glucose level increases, the number of bacteria also increases. Does the number of bacteria not continue to increase when the glucose concentration reaches a certain level?

12.“Excess glucose in murine models of diabetes increases the frequency of the fosfomycin resistant subpopulation”. The demonstration that this phenomenon occurs in two distinct murine models of diabetes (Type I and Type II) significantly strengthens the clinical relevance of the findings. It moves the mechanism from an in vitro observation to a potentially critical factor in treatment outcomes for a large patient population. Does the normal blood sugar concentration of the general population affect the number of drug-resistant subgroups?The in vivo correlation with hyperglycemia/diabetes is demonstrated in murine models. The translation of these findings to human infections, where the metabolic microenvironment may be more complex, requires clinical validation.

13. The single-cell data in Figures 2, 3, and 4 are compelling. Could the authors provide a quantitative measure of the heterogeneity (e.g., coefficient of variation) for PglpTmCherry expression under different conditions (e.g., +/- glucose) to supplement the frequency plots? This would further strengthen the description of the population shift.

14. Extended Data Figure 6 nicely shows the glucose effect in other clinical isolates. A brief comment in the main text or discussion emphasizing that this mechanism is not unique to Mu208 would be helpful to underscore its broader relevance.

Reviewer #3

(Remarks to the Author)
Choby et al. studied fosfomycin heteroresistance in one E. cloacae complex isolate and one susceptible control. The authors investigated the interplay of GlpT, fosA, and glucose on fosfomycin heteroresistance and tested the effects of host glucose levels on heteroresistance in murine models of infection with hyperglycemia. The manuscript is well written, with a clear language. Many supporting details are provided in the supplementary information. The manuscript figures are sufficiently clear, but the language used in the axis labels can be improved for clarity.
Minor comments:
105: What species within E. cloacae complex is Mu208? WGS data can be used for genomic species determination, e.g. with GTDBtk.
Across several figures: Be consistent when indicating percentages and proportions in axis labels, e.g. ‘Resistant to fosfomycin (%)’ vs. ‘% resistant to fosfomycin’. Could you improve labels such as ‘Frequency of population (%)’ e.g. to ‘Share of population (%)’?
493: How was the reference genome of Mu208 assembled?
724: define MHA

Add description for supplemental video

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript addressed most of the initial concerns either by additional experimentation or by highlighting the challenges that may hinder addressing some of the concerns. The following remain unaddressed or not fully addressed (numbered in relation to the original major concerns):

Major concern 6: The link to host metabolic state is among the most interesting and possibly clinically relevant findings of this study (which is also likely the reason this is referred to in the title); hence, it would require more robust proof. I thank the authors for clarifying the limitations/challenges. However, as it stands, there is not enough/robust evidence that an infection in the diabetes/hyperglycemina model would be worse than in a standard infection in non-diabetic mice. If the survival assays (similar to those in Fig 1e) for these diabetic models show even subtle survival shifts, these would be complementary to the shown CFU differences (which are relatively subtle themselves), further supporting the claims. An explicit mention of the limitations/challenges to demonstrate clearer link between the metabolic state and the treatment outcome due to these HR strains should be included. Alternatively, the authors could test another strain/species other than Mu208 in these assays. For example, is there a K. pneumoniae or other from those tested in Supp Fig 12 that may be more suitable to demonstrate the effect of host glucose (host disease state)?

Major concern 1: The edits and additional discussion mostly addressed major concern 1. However, a more explicit limitation statement (that the observed results could be due to the high level of resistance rather than HR per se) should be included in the main text and not only in the response to reviewers. Further, in the discussion added at line 463: “clinically relevant resistant subpopulation can exist as low as 0.0001% of the population” How was that assessed? There is no citation associated with that statement. This should be clarified and/or supported with a citation. Also, Line 470, “a population survive” could be possibly revised to “a population that survives”?

Reviewer #2

(Remarks to the Author)
The author has already addressed all the issues; it is recommended for acceptance.

REVIEWER COMMENTS

We thank the reviewers for their thoughtful reviews of our manuscript, which we feel have allowed us to significantly improve this work. As detailed below, we have added 17 new panels of data and made requested text changes. Please note that the Extended Data section in the original version has been formatted as Supplementary Figures, and line numbers refer to the tracked version of the combined manuscript.

Reviewer #1 (Remarks to the Author):
Work by Choby et al. explores the mechanism by which Enterobacter cloacae complex heteroresistance leads to treatment failure. Through a Tn-seq analysis, the fosfomycin resistant determinant FosA was identified as a requirement for high-level fosfomycin resistance (growth at 256 µg/mL). Other mechanistic studies, including elegant promoter-reporter assays, revealed variation in the expression of the gene encoding the glycerol-3-phosphate importer GlpT across the population, likely resulting in heteroresistance. The presence of glucose also decreased glpT expression, proposing a potential relevance of this phenotype in diabetic patients. Interesting, testing a type 1 and 2 diabetes in vivo models showed increased Mu208 CFUs growing at 256 ug/mL fosfomycin. The report is well written and describes an interesting observation of potential clinical relevance; however, some of the main conclusions lack support from the data currently presented.

Major concerns:

1. Fig 1d-e: Cause of in vivo treatment failure. The link between HR and failure cannot be established based on the presented results. A good proportion of the population is growing at 256 (the breakpoint, Fig 1a) so it is not clear if failure is due to HR or just overall higher level of resistance compared to the Mu819 strain susceptible to <32 ug/mL. Notably, in figures 1d and 1e, both strains were treated with the same fosfomycin concentration, but Mu819 is already susceptible, while Mu208 is resistant. Similarly, Figure 1i wild-type is resistant, and Figure 1j ΔgshA is susceptible, raising similar concern that the data would suggest that the treatment failure is due to resistance, not heteroresistance. Strains that have comparable fosfomycin MIC but differ in whether they are HR or not should be tested.

We thank the reviewer for this comment, and agree that the high frequency of resistant cells in Mu208 presents a challenge. By our definitions, any isolate which has cells surviving at the breakpoint is either HR or resistant. Mu208 is HR because only a fraction of the population survives at the breakpoint, rather than the entire population as observed in conventional, homogeneous resistance. Unfortunately, we do not have a homogeneously resistant strain that has a similar MIC to Mu208 as measured by conventional methods, since fosfomycin HR is the dominant phenotype of *Enterobacter* clinical isolates we have tested. If a comparison was possible for Mu208 (HR) versus a resistant isolate with the same MIC, we would expect both to cause treatment failure.

We recognize the limitations imposed by the features of Mu208, and have therefore edited line 135 to "However, fosfomycin treatment did not rescue mice infected with Mu208, demonstrating that a fosfomycin HR isolate can cause *in vivo* treatment failure (Figure 1e)." rather than the original phrase "fosfomycin HR can cause…"

We also added a section in the Discussion at line 463 to address this point:

"We investigated the mechanism of fosfomycin heteroresistance in *Enterobacter cloacae* complex strain Mu208, which caused fosfomycin treatment failure in a murine model of peritonitis (Figure 1e). 10% of the total cells of this isolate are resistant to the breakpoint concentration of 256 µg/mL (Figure 1a), which is relatively high considering that clinically relevant resistant subpopulations can exist as low as 0.0001% of the population. The Δ*gshA* mutant (lacking a glutathione synthetic gene and thus resulting in lower glutathione availability for the FosA resistance enzyme to inactivate fosfomycin) did not cause treatment failure; this isolate does have a population survive at the breakpoint and higher, but at a much lower frequency than the wildtype strain (Figure 1h), demonstrating that subpopulation frequency is likely a contributing factor to causing treatment failure. Whether a high frequency of cells surviving at the breakpoint or the existence of a subpopulation that can resist high concentrations of antibiotic (Mu208 has subpopulations that survive beyond 2048 µg/mL fosfomycin) is more critical to causing treatment failure is unclear. However, we can conclude that a heteroresistant strain can cause treatment failure."

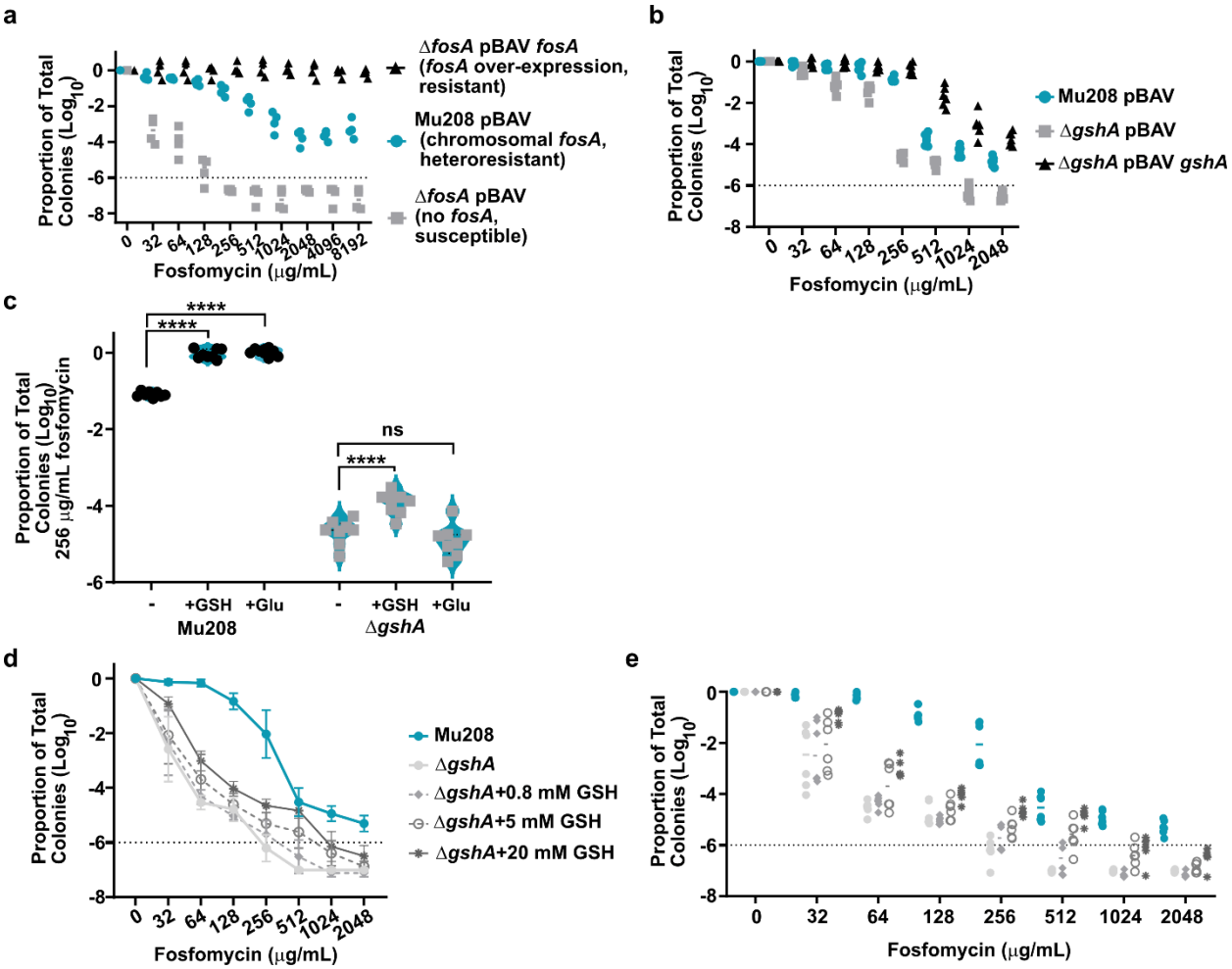
2. Fig 1j & extended Fig 2c: Interesting results but they do not necessarily mean that endogenous production is absolutely required but may have to do with the level of GSH in the host. In vitro, exogenous GSH does not chemically complement the WT phenotype (Extended Fig 2c) although that was performed at a single GSH concentration. There is a potential that the in vivo level of GSH in mice (or that exogenously supplemented in vitro) is not high enough relative to normal endogenous production. The chemical complementation experiment should be done in dose and higher levels of in vitro supplementation should be tested. Also, a discussion on the relative abundance of this metabolite in mice (& other mammalian models) and humans will then provide insights into whether the same effects may be observed in human or not - similar potential discrepancies were previously observed for certain vitamins and metabolites...

Thank you for the comment. We agree that chemical complementation does not fully rescue the resistant subpopulation in the Δ*gshA* strain. We have now performed a dose response in this mutant with 0.8, 5, and 20 mM glutathione. We are unable to use higher concentrations as additions greater than 20 mM severely inhibit growth. Related to point 4 below, we present the full population analysis profile for the glutathione addition in Supplementary Figure 4d-e.

We also added a section in the Discussion at line 476:

"We observed that the Δ*gshA* mutant, defective in glutathione (GSH) synthesis, had a reduced frequency of the fosfomycin resistant subpopulation (Figure 1h). Adding GSH to the media at very high concentrations, up to 20 mM, only modestly increased resistant subpopulation frequency in the Δ*gshA* mutant (Supplementary Figure 4c-e). This is consistent with data indicating that cytoplasmic GSH levels are challenging to complement exogenously[32,33] (FosA is cytoplasmic and thus GSH would need to reach the cytosol for use by this resistance enzyme). This may in part be due to the hydrolysis of periplasmic GSH by gamma-glutamyltranspeptidase GGT before it is imported to the cytosol[34]. In mammals, glutathione is generally measured at low millimolar concentrations intracellularly (1-8 mM[23,35]) but significantly lower levels extracellularly (2-3 µM[36,37] in plasma). Some extracellular niches have higher GSH concentrations: >400 µM in lung epithelial lining fluid[37] and 1-6 mM in bile[38,39]. Thus, we conclude that glutathione synthesis is critical for Mu208 to cause treatment failure, but

whether this is because this strain was unable to completely import the available GSH and provide it to FosA, or whether host sources of GSH were insufficient in quantity, remains unresolved."



Supplementary Figure 4. FosA and glutathione synthesis support the resistant subpopulation. (a) Alternative presentation of PAP of Mu208, the Δ*fosA* deletion strain, and its complement two independent experiments with 2 biological replicates each. (b) Alternative presentation of PAP of strains Mu208, Δ*gshA* mutant and its complement two independent experiments with 3 biological replicates each. (c) Proportion of population resistant to fosfomycin following growth in Mueller Hinton broth, with addition of 20 mM glutathione (+GSH) or 40 mM L-glutamic acid (+Glu) in strains Mu208 and Δ*gshA*, from two independent experiments with 4 biological replicates each. **** indicates $p<0.0001$ by one-way ANOVA with Šídák's multiple comparisons test, $F(5, 42) = 669.8$. (d-e) PAP of Mu208 grown in Mueller-Hinton broth (MHB), or Δ*gshA* mutant grown in MHB without or with glutathione (GSH) at concentrations indicated. (e) is an alternative presentation showing each data point, (d-e) are from two independent experiments with 3 biological replicates each.

3. Low frequency glpT null mutants: this small subpopulation described in Extended Fig 3d (first half of P. 8) appear to be enriched by the antibiotic, potentially accounting for – at least in part – for the

observed HR. It is not clear why this mechanism was largely dismissed in the subsequent sections exploring the different glpT expression levels. Importantly, how this subpopulation is accounted for in the mCherry experiments as these experiments do not consider the potential impacts of a null glpT mutation within the bacterial population. Controls addressing this should be included. Is there a potential for a dual mechanism or is one more dominant?

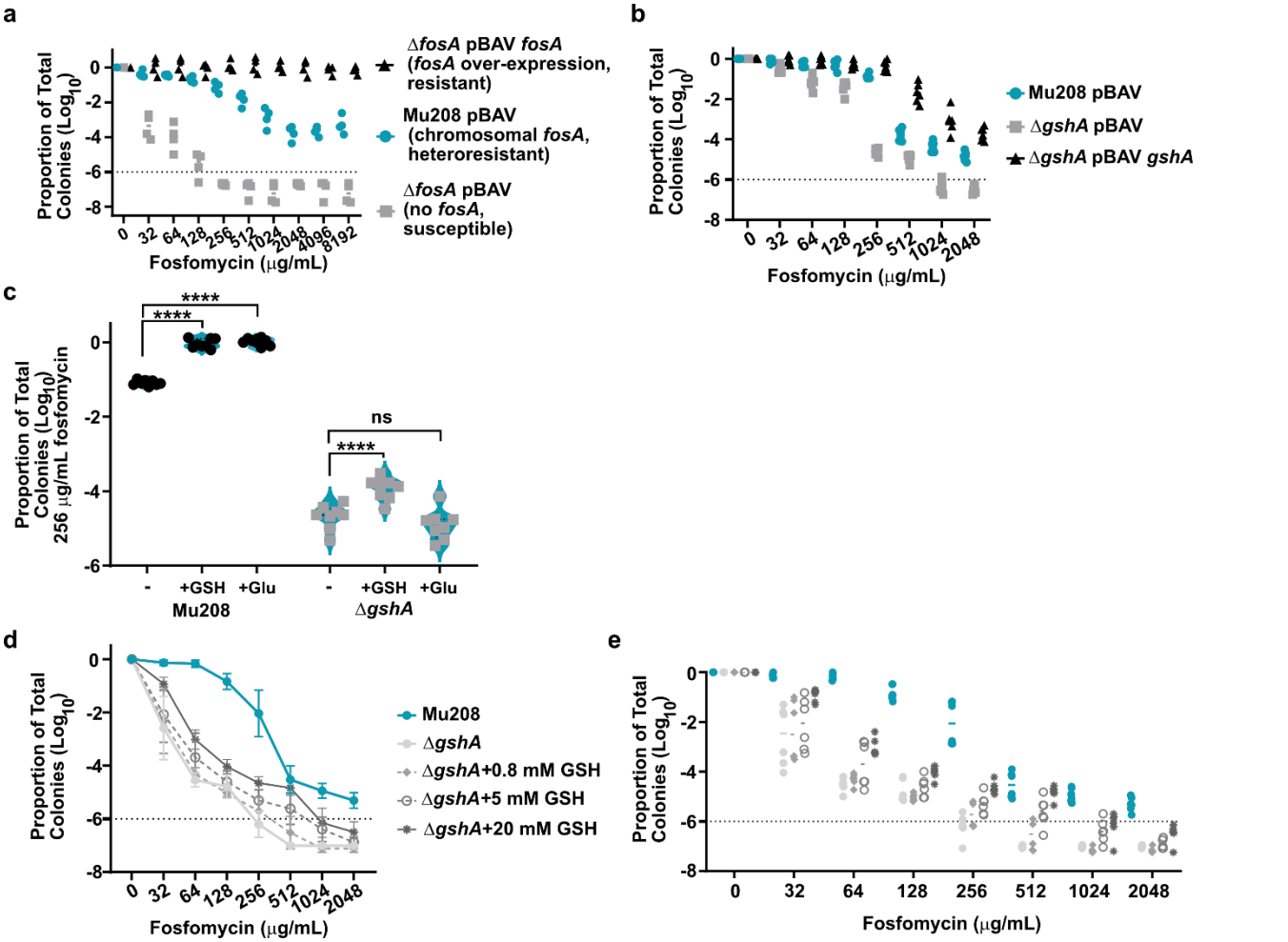
We appreciate this comment and have added text in the Discussion to address these questions, at line 500:

"Additionally, we did not expect to measure the fluorescence and survival of any *glpT* null mutants, as it is unlikely a cell which exists at 1 in 100,000 (Supplementary Figure 6c) would be visualized in an assay measuring 700-1000 cells. However, data presented here establishes that in Mu208, random, low frequency *glpT* inactivating mutants play a minor role in the resistant subpopulation. Following enrichment of the resistant subpopulation in broth, the population returned to baseline frequency when fosfomycin was removed, which confirmed that there was not a strong overall selection of stable resistant mutants (Figure 1c). Increasing expression of *glpT* by deleting *glpR*, which would not affect *glpT* null mutants, exerted a robust effect on the resistant subpopulation, consistent with *glpT* transcriptional regulation being a key driver of the resistant subpopulation (Figure 3b). When the subpopulation of wildtype Mu208 which survived at the breakpoint was assessed for *glpT* function, *glpT* null cells were determined to be present at a frequency of 1 in 10^5 (Supplementary Figure 6c). This rare subset of resistant cells only became dominant when sufficiently high concentrations of fosfomycin eliminated the vast majority of resistant cells with intact *glpT*. For wildtype Mu208, the resistant cell frequency dropped to 1 in 10^5 at 2048 µg/mL fosfomycin, and those surviving cells were mostly *glpT* null (Supplementary Figure 6d). This is consistent with the differing mechanisms of resistance; the vast majority of cells which survived fosfomycin had intact *glpT* but reduced expression. This population survived to 1024 µg/mL, and less so at 2048 µg/mL. *glpT* null cells likely had a higher single cell MIC due to complete *glpT* inactivation, and survived to at least 2048 µg/mL, but their very low frequency resulted in only a minor contribution to the composition of the total resistant subpopulation across the population."

4. HR vs resistance: The choice to use a single concentration for many assays, equivalent to CLSI breakpoint, (e.g., Fig 3d, 4a, c, d, and others including in vivo exp in Fig 5) does not capture subpopulation level frequencies and may just reflect MIC shifts (i.e., resistance). In general, heterogeneity cannot be as conclusive by testing at a single concentration only. More concentrations, including sub-breakpoint ones, should be tested. That includes adding a few representative dose-response PAP-like assays for the created reporters.

Thank you for the comment. In our experience, a shift in subpopulation frequency at the breakpoint is associated with shifts at additional concentrations. However, for the sake of thoroughness, we have repeated many of these assays to include a full PAP, reproduced below: for glutathione addition (Supplementary Figure 4d-e), cyclic AMP (Supplementary Figure 9e-f), glucose addition to Mueller Hinton broth (Supplementary Figure 10a-b), and representative glucose additions to M9 glycerol (Supplementary Figure 10c-d).

We have also performed full PAP with each fluorescent reporter, and added the text lines 323 "Importantly, all reporters phenocopied their respective parental strain in fosfomycin PAP (Supplementary Fig. 8b—d)."



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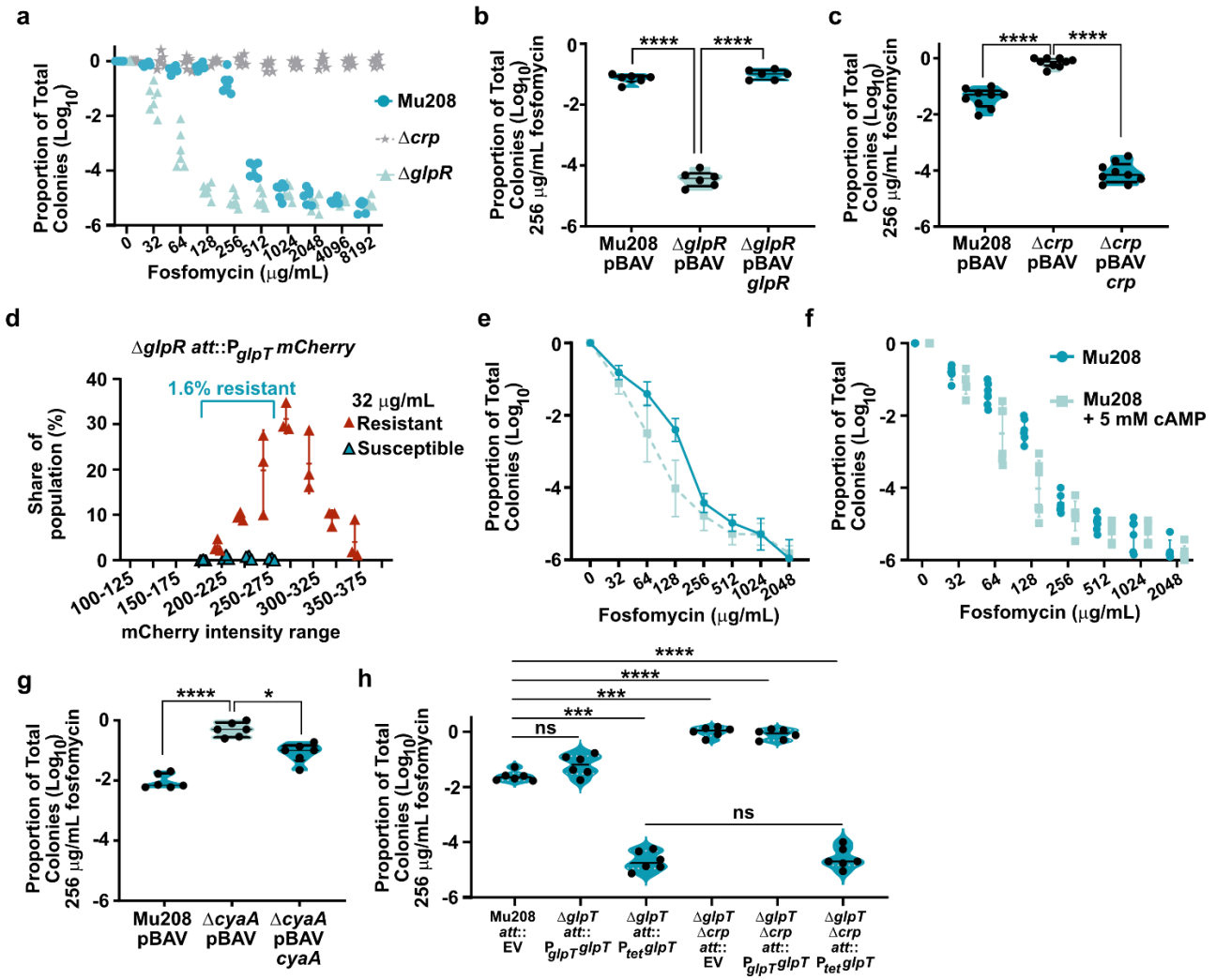
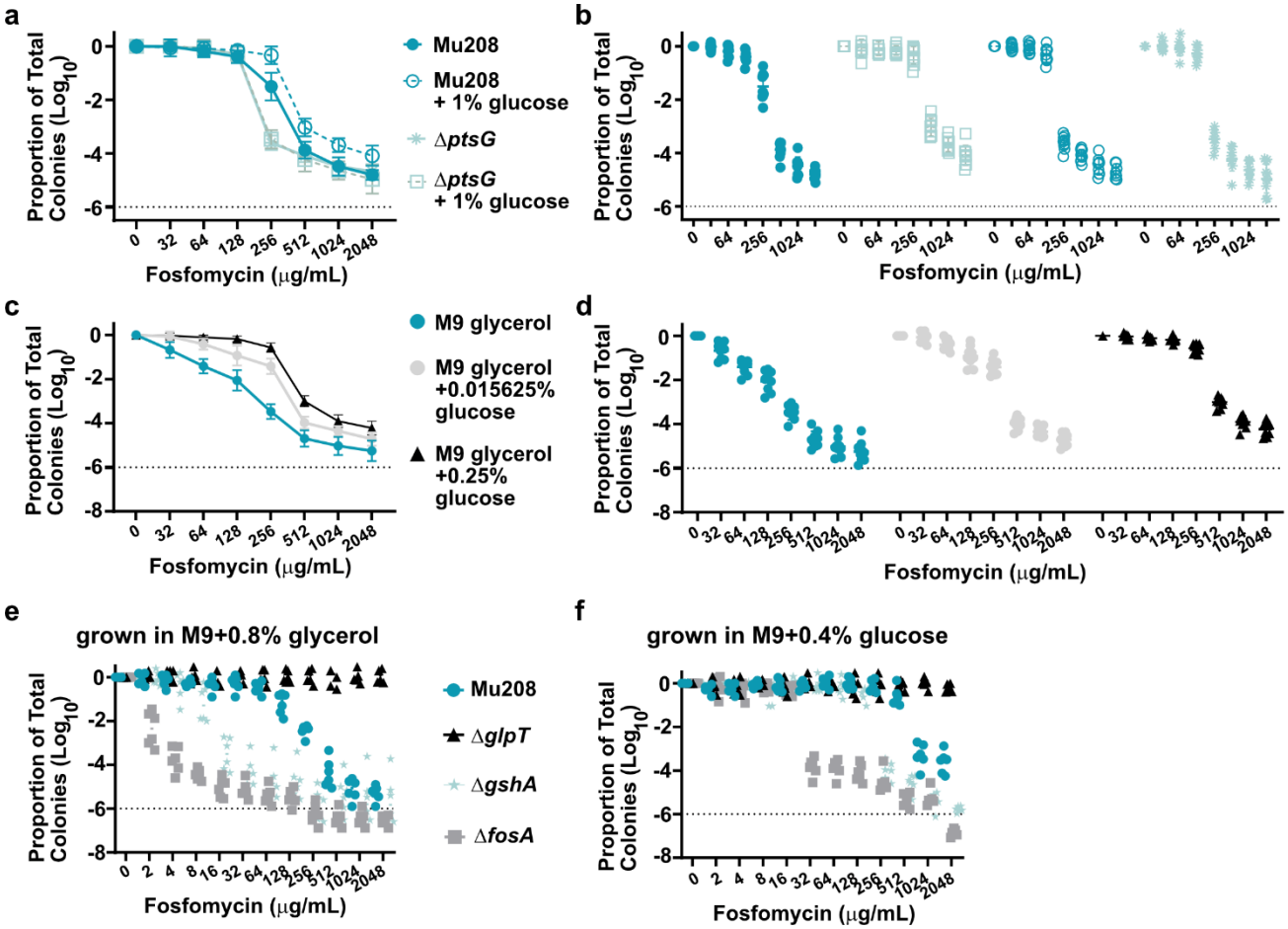
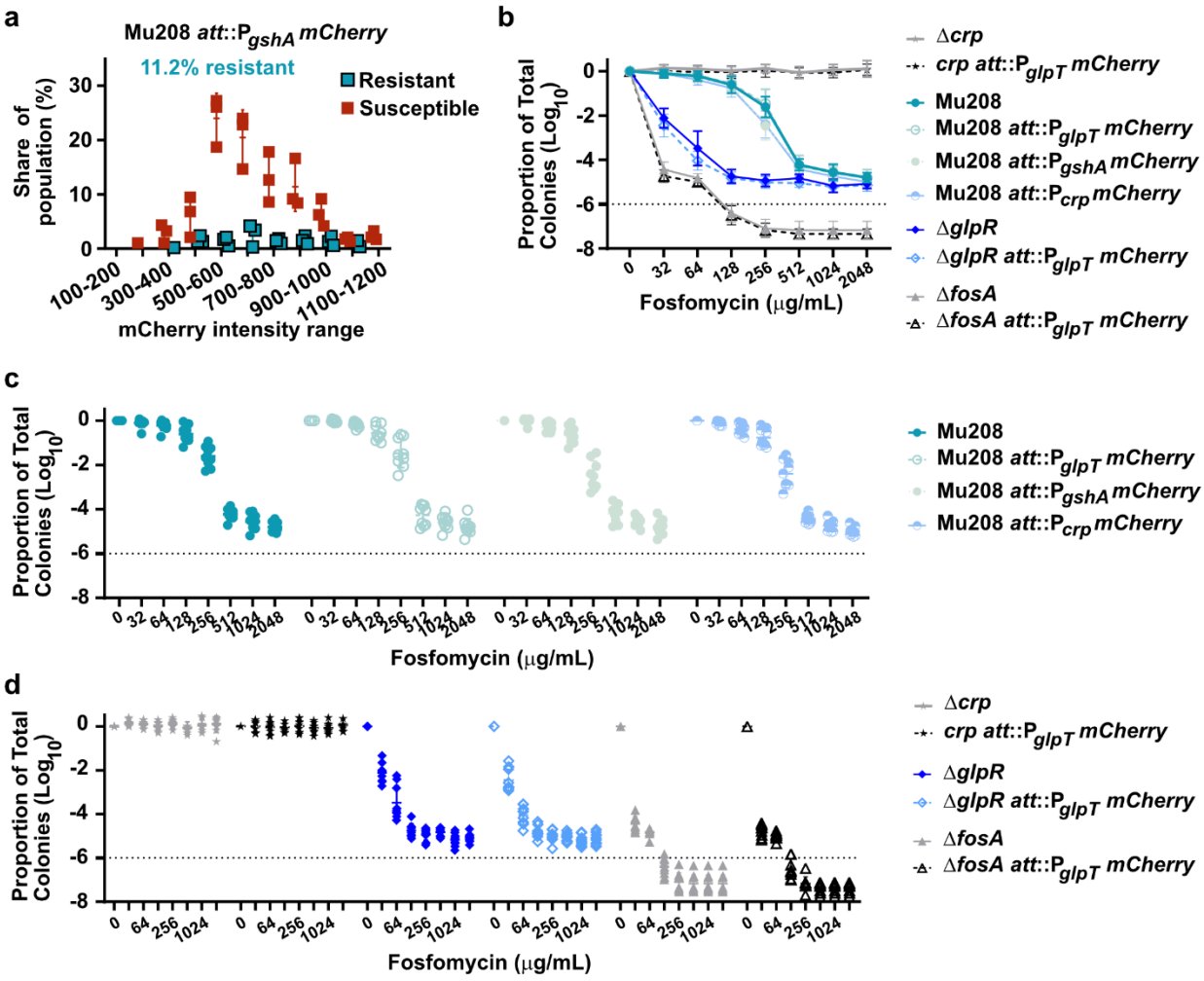


Supplementary Figure 9. Regulation of *glpT* by GlpR and CyaA/CRP controls the subpopulation. (a) Alternative presentation of population analysis profile (PAP) for strains Mu208, Δ*cyaA*, Δ*crp*, and Δ*glpR*, from Figure 3a, from two independent experiments with 3 biological replicates each. (b-c) Proportion of colonies resistant to fosfomycin in strains Mu208, mutants, and their complement, from two independent experiments with 3 biological replicates each. **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, b: F (1.760, 8.799) = 799.0, c: F (1.208, 9.665) = 293.3. ((d)) P_{glpT}*mcherry* expression intensity in the Δ*glpR* mutant among cells that survived or were killed by 32 μg/mL fosfomycin, from three independent experiments; data were determined from 700-1000 cells for each experiment. (e-f) PAP of Mu208 following growth in in M9+0.8% glycerol broth containing 0 or 5 mM cAMP. From two independent experiments with 3 biological replicates each. (f) is an alternative presentation showing each replicate. (g) Proportion of colonies resistant to fosfomycin in strains Mu208, Δ*cyaA* mutant, and the complement, from two independent experiments with 3 biological replicates each. * indicates p= 0.0127 and **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, d: F (1.411, 7.055) = 55.80 (g) Proportion of colonies resistant to fosfomycin in strains indicated (EV indicates empty vector integrated into *att* site, from two independent experiments with 3 biological replicates each. ns indicates p>0.05, *** indicates p= 0.0003 or 0.0001 (left to right) and **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, F (2.748, 13.74) = 319.7.



Supplementary Figure 10. Glucose enhances the resistant subpopulation in Mu208. (a-b) PAP of Mu208 and ΔptsG mutant following growth in MHB or MHB+1% glucose, **(b)** is an alternative presentation of panel a; **(a-b)** are from three independent experiments with 3 biological replicates each. **(c-d**) PAP of Mu208 following growth in M9+0.4% glycerol as the sole carbon source or glycerol and glucose as indicated. **(d)** is an alternative presentation of panel c. **(c-d)** are from three independent experiments with 3 biological replicates each. **(e-f)** Alternative presentation of population analysis profiles from strains Mu208, Δ*glpT*, Δ*gshA*, and Δ*fosA*. **(e)** following growth in M9+ glycerol as the sole carbon source or **(f)** following growth in M9+glucose as the sole carbon source, from two independent experiments with 3 biological replicates each.



Supplementary Figure 8. P*gshA*-*mcherry* is not a predictor of survival, and reporter strains phenocopy parental strains. (a) P*gshA*-*mcherry* expression intensity among cells that survived or were killed by 128 μg/mL fosfomycin, from three independent experiments; data were determined from 700-1000 cells for each experiment. **(b)** PAP of Mu208, mutants, and strains encoding *mCherry* reporters **(c-d)** Alternative PAP presentation of panel (b). **(b-d)** are from three independent experiments with 3 biological replicates each.

5. mCherry promoter reporter assays limit of detection. While these reporter assays offer a powerful approach to determine cell level expression and phenotypic effects, the limit of detection of this assay seems high compared to the PAP analysis. For example, if the frequency of a resistant subpopulation in 1 in 100,000, the promoter reporter will not be able to capture this subpopulation with the current assay design, which only includes 700 - 1000 cells. This is evident in the discrepancy between Figure 3b ΔglpR and Figure 3c, where there is a subpopulation surviving at 128 μg/mL fosfomycin in Figure 3b, but in Figure 3c, there is no resistant population. Testing more concentrations, as mentioned above, may partly mitigate this concern and a discussion about the limitation of this assay should be included.

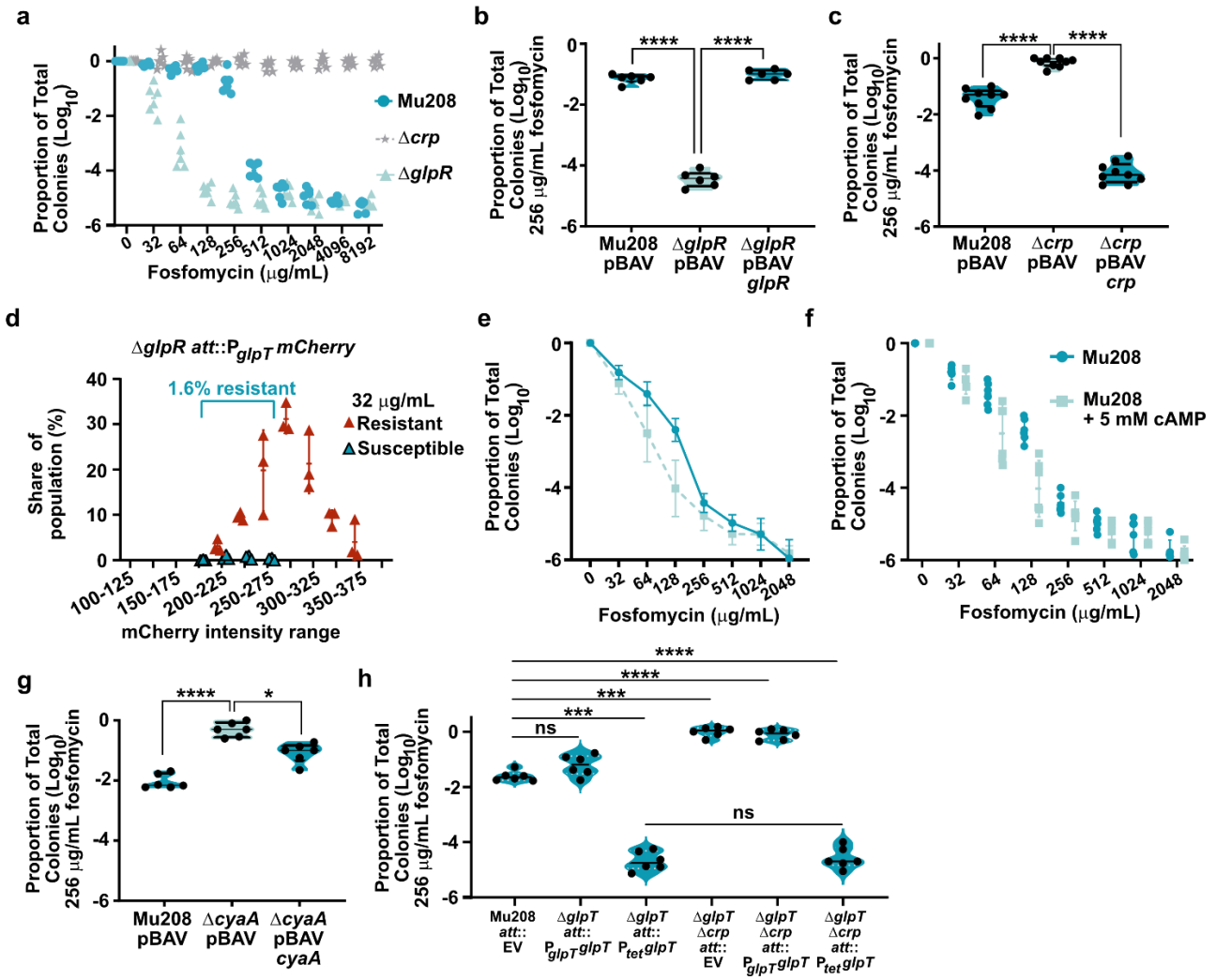
We appreciate this comment. We performed the imaging experiment with the Δ*glpR* strain as suggested, at 32 μg/mL fosfomycin. We edited the text at line 299:

“At lower fosfomycin concentrations, the Δ*glpR* strain exhibited resistant cells (Figure 3b). Therefore, we repeated the imaging at 32 µg/mL fosfomycin, where some cells survived, and again determined that surviving cells had lower P_{glpT}*mcherry* activity as compared to susceptible cells (Supplementary Fig. 9d).”

Supplementary Figure 9 is reproduced below.

We have also added a discussion of the limitations of this technique, at line 489

“Despite encoding FosA, the majority of the cells were killed by the breakpoint concentration of fosfomycin. We observed that most of the cells were susceptible at this concentration because they expressed sufficient GlpT, a glycerol importer that also imports fosfomycin. Mu208 was amenable to single-cell microscopy because the resistant subpopulation frequency was relatively high at the breakpoint concentration, but it is worth noting that imaging assays, with 700-1000 cells quantified, have a much higher limit of detection than the population analysis profile which detects killing or survival of ~10^7 cells. Mutant strains lacking genes involved in fosfomycin resistance were assessed by single-cell microscopy at lower drug concentrations where the frequency of cells resilient to fosfomycin was high enough to overcome the high limit of detection (Δ*fosA* in Figure 2e, Δ*glpR* in Figure 3c, Supplementary Figure 9d). Despite these limitations, examining *glpT* expression and cell fate with single-cell resolution provided critical insights into the means of survival of the resistant subpopulation.”



Supplementary Figure 9. Regulation of *glpT* by GlpR and CyaA/CRP controls the subpopulation. (a) Alternative presentation of population analysis profile (PAP) for strains Mu208, Δ*cyaA*, Δ*crp*, and Δ*glpR*, from Figure 3a, from two independent experiments with 3 biological replicates each. (b-c) Proportion of colonies resistant to fosfomycin in strains Mu208, mutants, and their complement, from two independent experiments with 3 biological replicates each. **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, b: F (1.760, 8.799) = 799.0, c: F (1.208, 9.665) = 293.3.. (d) P_{glpT}*mcherry* expression intensity in the Δ*glpR* mutant among cells that survived or were killed by 32 µg/mL fosfomycin, from three independent experiments; data were determined from 700-1000 cells for each experiment. (e-f) PAP of Mu208 following growth in in M9+0.8% glycerol broth containing 0 or 5 mM cAMP. From two independent experiments with 3 biological replicates each (f) is an alternative presentation showing each replicate. (g) Proportion of colonies resistant to fosfomycin in strains Mu208, Δ*cyaA* mutant, and the complement, from two independent experiments with 3 biological replicates each. * indicates p= 0.0127 and **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, d: F (1.411, 7.055) = 55.80 (g) Proportion of colonies resistant to fosfomycin in strains indicated (EV indicates empty vector integrated into *att* site, from two independent experiments with 3 biological replicates each. ns indicates p>0.05, *** indicates p= 0.0003 or 0.0001 (left to right) and **** indicates p<0.0001 by RM one-way ANOVA with Dunnett's multiple comparisons test, F (2.748, 13.74) = 319.7.

6. In vivo diabetes experiments. Fig 5: This is an interesting experiment. However, it should be complemented with testing the effect of fosfomycin on the therapeutic outcome (or survival) of these diabetic mice relative to control, especially that the CFU differences are within the same log.

We agree with the reviewer and have sought an opportunity to test whether the diabetic environment worsens treatment outcome with fosfomycin therapy. Because Mu208 already causes treatment failure (discussed above, Figure 1e), it would be challenging to observe that the diabetic environment caused 'worse' failure than what we already observed. Ideally, we would have a strain of *Enterobacter* that is susceptible when grown without glucoses but becomes HR following growth with glucose to test this hypothesis in vivo. Unfortunately, we have not identified such a strain. Future efforts will address this if a suitable clinical isolate is identified. In addition, we hope to determine in the future whether diabetes is associated with worsened outcomes of fosfomycin therapy in humans.

7. Heteroresistance definition. The authors refer to prior literature that offer standard definitions of HR but adapt them through four criteria. Notably, this group places emphasis on clinical breakpoints. While this is understandable to infer clinical relevance to the phenotypes, there should be a discussion acknowledging that the specific breakpoint value may change over time (as occasionally done by CLSI and others). Also, the criteria should align with similar studies from the same group number (such as https://doi.org/10.1038/s41467-025-60828-9) by removing reference to 2X breakpoint in the second criterion related to Level of resistance.

Thank you. We have removed the reference to 2X breakpoint in our definition, and have added the following text at line 662:

"We include the breakpoint in our definition of HR because we are interested in the clinical relevance of this phenomenon beyond the biological questions posed by the existence of heterogeneous subpopulations. It is worth noting that breakpoints can change based on clinical and regulatory changes, and occasionally breakpoints differ between regulatory agencies. Nevertheless, the inclusion of the breakpoint in our definition defines subpopulations that are resistant to clinically relevant antibiotic concentrations."

Minor comments

a) The first section in the Results section does not describe the investigation of the mechanism of fosfomycin HR. Therefore, the first sentence in this section should be revised accordingly.

Thank you. We have revised the first sentence at line 114 as

"We assessed the features of fosfomycin heteroresistance (HR) in a clinical isolate of the *Enterobacter cloacae* complex, *E. kobei* strain Mu208."

b) Page 8, line 1: the data presented thus far in the manuscript can perhaps "hypothesize" but not "support" the model yet. Please revise this sentence.

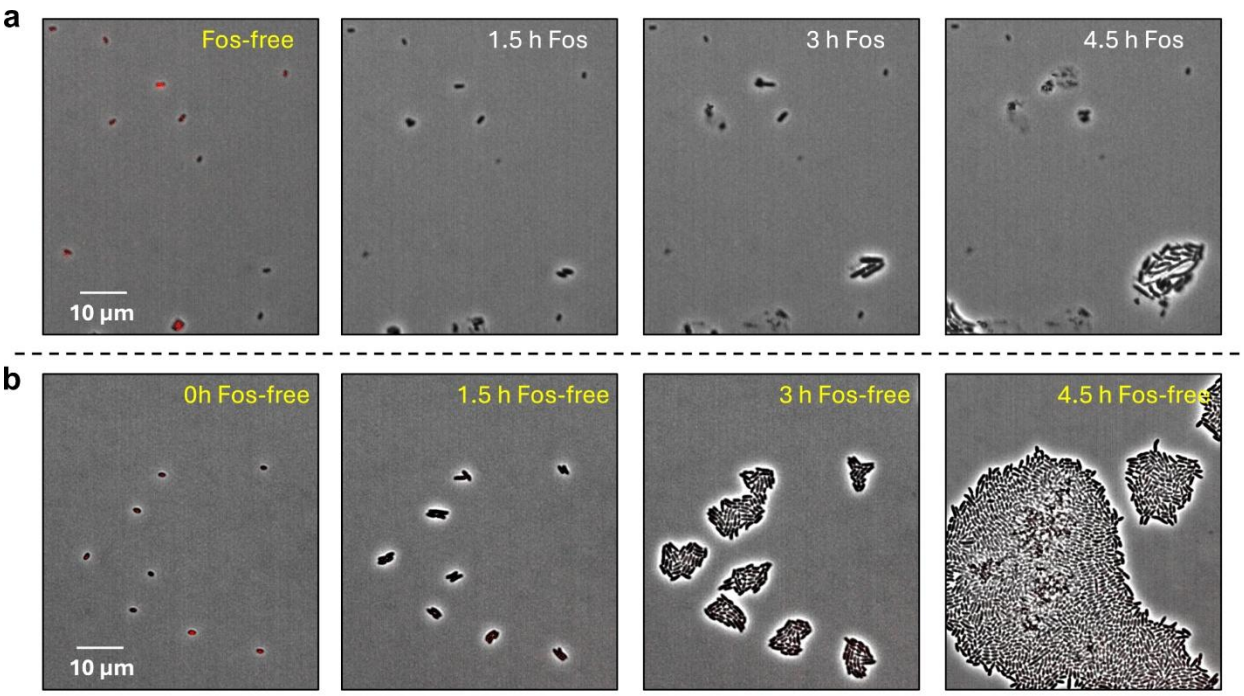
We have replaced "model" with "hypothesis".

c) For Figure 2d, the figure displays 256 µg/mL of fosfomycin while the caption says 128 µg/mL.

We appreciate identification of this error. The figure has been corrected.

d) Fig 2c: please show more representative images (in supp or extended data fig) of this (glpT) and the control esp. for panels with mCherry expressed.

We have added additional representative images in Supplementary Figure 7, as well as imaging in the absence of fosfomycin. We edited the text at line 260 "In the absence of fosfomycin, all cells replicate uniformly regardless of initial P_{glpT}*mcherry* activity (Supplementary Fig. 7b)."



Supplementary Figure 7. Mu208 *att*:: P_{glpT}*mCherry* expression affects growth in fosfomycin but not in the absence of fosfomycin. (A) Additional representative microscopy images associated with Figure 2c. of Mu208 *att*:: P_{glpT}*mCherry* on agar pads in the absence of fosfomycin, followed by images of the time-lapse following fosfomycin addition. See also Supplementary Video **(B)** Growth progression of Mu208 *att*:: P_{glpT}*mCherry* in the absence of fosfomycin.

Reviewer #2 (Remarks to the Author):
Reviewer's comments:
This is an outstanding and comprehensive study that elucidates a novel mechanism of fosfomycin heteroresistance in Enterobacter cloacae. The authors convincingly demonstrate that heterogeneity in the expression of the fosfomycin importer GlpT, regulated by host metabolic cues (specifically glucose), tunes the frequency of the resistant subpopulation. The combination of this heterogeneity with the "resistance enhancer" FosA drives the heteroresistant phenotype. The translation of this finding into murine models of hyperglycemia/diabetes, showing a clinically relevant increase in the resistant subpopulation in vivo, is a significant strength and highlights the potential impact of host physiology on antibiotic treatment outcomes. The manuscript is well-written, the data are robust and support the conclusions. However, several critical issues must be addressed before the manuscript is suitable for publication.

We thank the reviewer for the thorough and positive feedback.

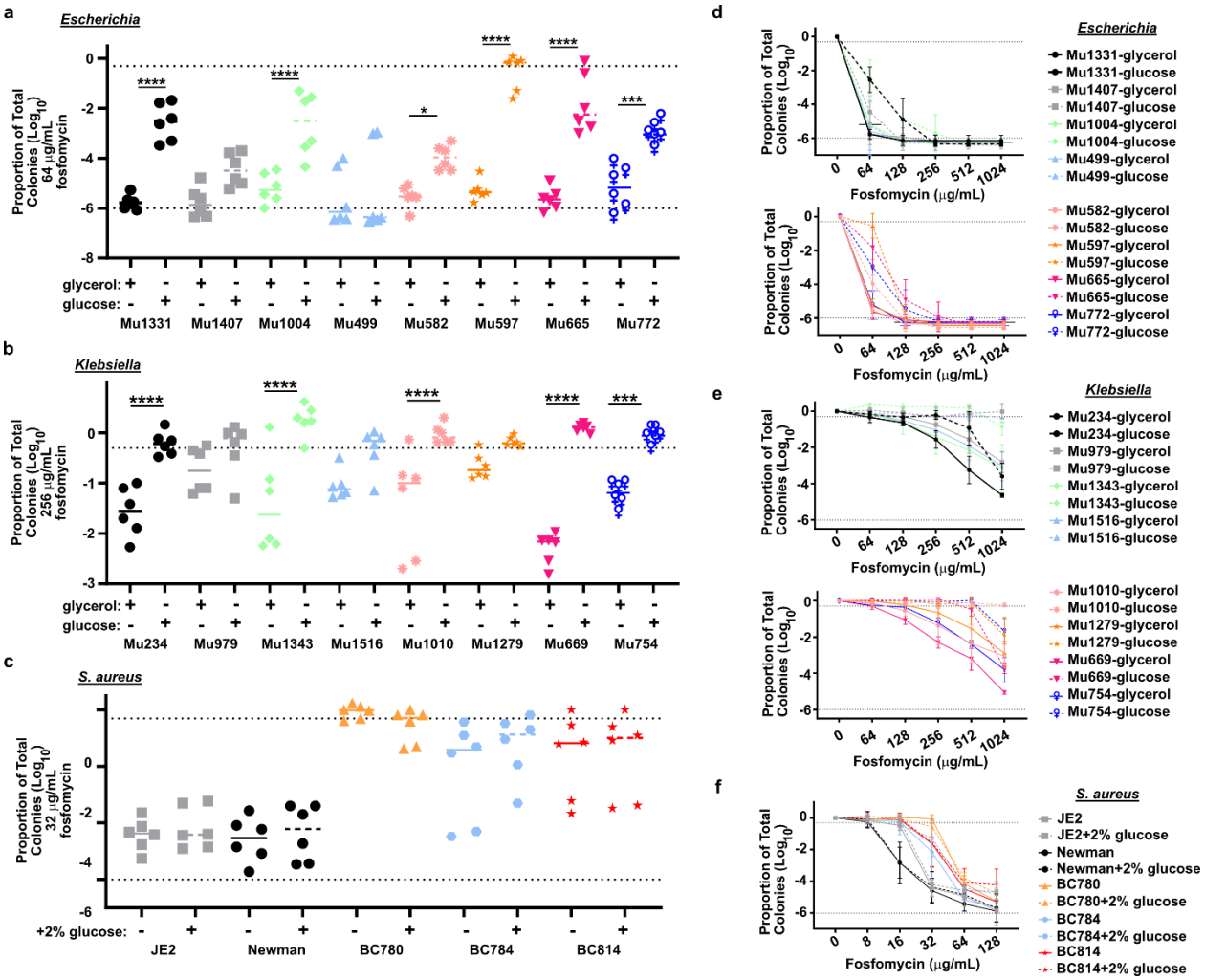
Major issues:
1.The paper introduces the paradigm-shifting concept that the host's metabolic environment (glucose levels) can directly modulate the frequency of an antibiotic-resistant Enterobacter cloacae subpopulation (fosfomycin resistant) during infection and author suggested that the metabolic context of infection in specific disease states should be considered when prescribing antibiotics. What I'm curious

about is whether other bacteria such as the E.coli, Klebsiella, or Staphylococcusalso have a similar mechanism.

We appreciate this comment and have conducted experiments to test whether glucose enhanced fosfomycin resistance in *E. coli, Klebsiella,* and *Staphylococcus*. As shown in Supp. Fig. 12, reproduced below, growth in glucose enhances the ability of resistant CFU to form on fosfomycin across most carbapenem-resistant *Escherichia* (of which most are *E. coli*) and *Klebsiella* isolates we tested. Consistent with literature demonstrating that most *E. coli* are fosfomycin susceptible and do not encode *fosA*, *Escherichia* isolates are all fosfomycin susceptible by PAP. However, the resistant subpopulation at sub-breakpoint concentrations is enhanced by glucose. In fosfomycin-HR *Klebsiella* isolates, growth in glucose resulted in complete resistance at 256 µg/mL fosfomycin. Finally, we tested a group of *S. aureus* isolates and found that glucose had no effect on fosfomycin resistance measured by PAP.

We have revised the text to include these new results as follows, at line 388:

"We next sought to test whether glucose was able to enhance the frequency of the resistant subpopulation among carbapenem-resistant *Enterobacter* clinical isolates. We grew four additional isolates collected by the Georgia Emerging Infections Program (GaEIP) in Mueller-Hinton broth with or without additional glucose (Supplementary Fig. 11a) or in M9 with either glycerol or glucose as the sole carbon source (Supplementary Fig. 11b). In both, glucose resulted in increased frequency of the population resistant to 256 µg/mL fosfomycin. Next, we performed PAP of 8 GaEIP isolates each of carbapenem-resistant *Escherichia* and *Klebsiella* following growth in M9 media with glycerol or glucose as the sole carbon source. *Escherichia* isolates were susceptible by PAP, consistent with infrequent carriage of the *fosA* resistance gene[14]. However, six of the eight *E. coli* isolates demonstrated a significant increase in the proportion of the population resistant to 64 µg/mL fosfomycin when grown in glucose as compared to glycerol (Supplementary Fig. 12a, d). While *Klebsiella* isolates were either HR or resistant to fosfomycin, five of eight showed a significant increase in the proportion of the population resistant to 256 µg/mL fosfomycin when grown in glucose (Supplementary Fig. 12b, e). In contrast, when we performed PAP of *Staphylococcus aureus* isolates (model strains Newman and JE2, and three previously described clinical isolates[30] following growth in Mueller Hinton broth with or without additional 2% glucose added, we observed that glucose did not enhance the proportion of the population resistant to 32 µg/mL fosfomycin (Supplementary Fig. 12c, f). Together, our data support the hypothesis that glucose can generally increase relative fosfomycin resistance in *Enterobacter*, *Escherichia*, and *Klebsiella*."



Supplementary Figure 12. Glucose enhances the frequency of the fosfomycin resistant subpopulation in *Escherichia* and *Klebsiella* but not *S. aureus*. (a-d) *Escherichia* or *Klebsiella* isolates were grown in M9 media with 0.8% glycerol or 0.4% glucose as the sole carbon source and **(a,c)** PAP was performed. The proportion of colonies resistant to **(b)** 64 µg/mL fosfomycin or **(d)** 256 µg/mL fosfomycin are presented from the same experiments. **(e-f)** *S. aureus* isolates were grown in Mueller Hinton broth ±2% glucose and **(e)** PAP was performed. **(f)** The proportion of colonies resistant to 32 µg/mL fosfomycin is presented. **(a-f)** are from three independent experiments with 2 biological replicates each. In **(b)**, * indicates p=0.0151, *** indicates p=0.0001, and **** indicates p<0.0001 by one-way ANOVA with Sidak's correction for multiple comparisons, F (15, 80) = 22.69. In **(d)**, *** indicates p=0.0003, and **** indicates p<0.0001 by one-way ANOVA with Sidak's correction for multiple comparisons, F (15, 80) = 14.97. In **(f)**, no significance was detected by one-way ANOVA with Sidak's correction for multiple comparisons F (9, 50) = 15.50.

2." we observed that the resistant subpopulation was more frequent during infection in murine models of hyperglycemia/diabetes". How about the clinical -derived beacterial that isolated from the diabetic, or other patients. It is suggested to conduct more research on the heterogeneity of resistance of clinical strains to fosfomycin. Whether the mechanisms of heterogeneity resistance of these clinical strains to fosfomycin are all universal.

We agree that future work on fosfomycin HR, and heteroresistance in general, should better connect phenotypic in vitro testing to the patient source and patient status. Ideally we would be

able to directly quantify fosfomycin resistance and subpopulation frequency out of healthy control and diabetic patients before the isolates are cultured, but we do not have access to such samples. However, the effects of glucose on Enterobacterales is independent of the patient source, whether diabetic or not.
We have previously found that fosfomycin heteroresistance is much more common than conventional resistance in CRE, described in line 74, below. It is likely that many of these isolates were derived from diabetic patients, but we do not have sufficient clinical data.

"Fosfomycin HR is widespread in carbapenem-resistant Enterobacterales (CRE); we previously screened CRE clinical isolates through the Georgia Emerging Infections Program's Multi-site Gram-negative Surveillance Initiative and observed that fosfomycin HR was detected at a greater rate (72% of isolates) than HR to any other antibiotic tested[8]."

In response to the previous point, we have found that among Klebsiella HR isolates, glucose has a significant effect on the resistant subpopulation, suggesting that the mechanism we describe in Mu208 is conserved among CRE.

3.Mu208 is the Enterobacter kobei clinical isolate while MU819 is Enterobacter cloacae clinical isolate. What are the differences between these two types of bacteria? Will this affect their antimicrobial phenotypes?

Mu819 is *E. cloacae* according to clinical identification, but its species identity has not taxonomically verified. The *Enterobacter cloacae* complex has been recently undergoing frequent taxonomic revisions. As such, we have clarified by adjusting its description to "*Enterobacter cloacae* complex clinical isolate".

Proksee FastANI calculated average nucleotide identity between the chromosomes of Mu819 and Mu208 at ~88%. Using the Resistance Gene Identifier tool in The Comprehensive Antibiotic Resistance Database, both isolates encode a chromosomal *ampC* in the ACT family of β-lactamases, and antibiotic efflux genes (*baeRS, adeF, rsmA, emrB, marA, oqxA, kpnEF, msbA, acrA, leuO*). Most importantly, Mu208 encodes *fosA*, and we chose Mu819 because, unlike most carbapenem-resistant Enterobacter, Mu819 does not encode *fosA*.

4.Enterobacter cloacae strain and treated them with antibiotics to investigate the possible treatment failure caused by heterogeneous strains. However, we know that Enterobacter cloacae is usually a conditionally pathogenic bacterium and generally does not cause disease in normal mice. Did the author evaluate the pathogenicity of the mice?

We agree with the reviewer that Enterobacter is generally an opportunistic pathogen associated with immunocompromised hosts or those undergoing medical treatment. We selected our inocula based on preliminary experiments such that sufficient bacteria were injected to allow organ dissemination and colonization without rapid clearance. We have not evaluated pathogenesis of these isolates, beyond our observation that Mu208 and Mu819 are capable of causing death (reached humane endpoints) when untreated with antibiotics, as shown in Figure 1d, 1e, 1i, 1j.
We have revised the text to provide more context to this pathogen at line 84:

"The *Enterobacter cloacae* complex comprises a group of species with intrinsic and frequently acquired antibiotic resistance and the capacity to cause various infections. Generally, these species exist in a variety of environmental habitats and can colonize the

mammalian gut. However, they can be opportunistic, nosocomial pathogens of patients who are immunocompromised or otherwise more susceptible to infection. Relatively little is known mechanistically about the virulence factors and pathogenesis of these organisms[15]. Globally, *Enterobacter* spp. were the 7th most frequent cause of death by bacterial pathogens, generally from lower respiratory infections, bloodstream infections, and peritoneal and intra-abdominal infections[16]. Treatment options for multidrug resistant *Enterobacter* infections are limited, contributing to the Centers for Disease Control's designation of extended-spectrum β-lactamase (ESBL)-producing Enterobacterales as a Serious Threat and carbapenem-resistant Enterobacterales an Urgent Threat[17]. Thus, a greater understanding of antibiotic resistance and heteroresistance is critical to improving the capacity to treat infections caused by antibiotic-resistant *Enterobacter*."

5."Mu208 exhibited fosfomycin HR, harboring a subpopulation of 1-10% of cells resistant to 256 μg/mL fosfomycin and greate" It best known that when antibiotics are used, they can kill the sensitive bacteria while increasing the number of drug-resistant bacteria. How was this data calculated?

Thank you for the comment. We have clarified our description of the population analysis profile to better explain that the populations are resistant prior to fosfomycin exposure. The data in Figure 1b-c better demonstrate the ability of antibiotic to select for the resistant population. Revised text is at line 114, below:

"We assessed the features of fosfomycin heteroresistance (HR) in a clinical isolate of the *Enterobacter cloacae* complex, *E. kobei* strain Mu208. We used population analysis profile (PAP; Supplementary Fig. 1) to measure HR, which is performed by growing a bacterial strain in broth culture without antibiotic, then plating a range of dilutions of the culture on agar containing a range of concentrations of fosfomycin. Cells that are resistant to the antibiotic generate colonies on fosfomycin plates and are enumerated as colony forming units (CFU) at each concentration so that a proportion of the total population which survives at a given concentration can be calculated. Thus, PAP assesses resistant cells in the population prior to antibiotic exposure."

6."Therefore, the fosfomycin resistant subpopulation requires FosA and its cofactor glutathione" ," To test whether bacterial-derived glutathione is required even in the presence of mammalian-derived glutathione, we infected mice with a lethal dose of the ΔgshA glutathione synthase mutant, and found that this strain did not cause treatment failure, Thus, synthesis of the FosA cofactor glutathione is critical to the fosfomycin resistant subpopulation and the ability of strain Mu208 to cause fosfomycin treatment failure in vivo". I'd like to know the results of the overexpression strain of gshA.

We thank the reviewer for this comment. Because Mu208 already caused treatment failure (Figure 1e), it would be challenging to observe that the infection with Mu208 pBAV *gshA* caused 'worse' failure than what we already observed. We have validated the *gshA* mutant by complementation (Figure 1h).

Related, we have added more information and context to mammalian glutathione in the Discussion at line 476:

"We observed that the Δ*gshA* mutant, defective in glutathione (GSH) synthesis, had a reduced frequency of the fosfomycin resistant subpopulation (Figure 1h). Adding GSH to the media at very high concentrations, up to 20 mM, only modestly increased resistant subpopulation frequency in the Δ*gshA* mutant (Supplementary Figure 4c-e). This is consistent with data indicating that cytoplasmic GSH levels are challenging to complement exogenously[32,33] (FosA is cytoplasmic and thus GSH would need to reach the cytosol for use by this resistance enzyme). This may in part be due to the hydrolysis of periplasmic GSH by gamma-glutamyltranspeptidase GGT before it is imported to the cytosol[34]. In mammals, glutathione is generally measured at low millimolar concentrations intracellularly (1-8 mM[23,35]) but significantly lower levels extracellularly (2-3 μM[36,37] in plasma). Some extracellular niches have higher GSH concentrations: >400 μM in lung epithelial lining fluid[37] and 1-6 mM in bile[38,39]. Thus, we conclude that glutathione synthesis is critical for Mu208 to cause treatment failure, but whether this is because this strain was unable to completely import the available GSH and provide it to FosA, or whether host sources of GSH were insufficient in quantity, remains unresolved."

7"glpT inactivation confers fosfomycin resistance"and "Thus, there is a rare subpopulation of resistant cells with null glpT function, but the majority of the resistant subpopulation has intact GlpT" . Here is really confusion.

We apologize for being unclear. We have added to and edited the text at line 228:

"Our data support a hypothesis in which GlpT is the primary fosfomycin importer in Mu208. Genetic inactivation of *glpT* conferred homogenous resistance, presumably because fosfomycin entry into the cell is severely diminished. Mutations which ablate the expression or function of GlpT could represent a route to fosfomycin resistance in our assays. However, we hypothesized that most of the fosfomycin resistant population were not *glpT* mutants, because the frequency of the resistant subpopulation was much greater than the expected mutation rate (Figure 1a), the resistant subpopulation rapidly returned to baseline frequency when fosfomycin was removed suggesting this is a flexible phenotypic resistance trait rather than being caused by a more stable form of resistance such as mutation (Figure 1c), and because the resistant subpopulation, if generated entirely by random inactivating mutations to *glpT*, would exist at the same frequency across fosfomycin concentrations.

Nevertheless, we evaluated the resistant subpopulation in Mu208 for changes in *glpT* function by sequencing or phenotypic testing. We sequenced colonies collected from agar containing fosfomycin and performed variant analysis. We only found a mutation in *glpT* or its promoter in 1/7 biological replicates (Supplementary Fig. 6b). Phenotypically, mutations in *glpT* render bacteria unable to grow on glycerol phosphate as the sole carbon source, as it cannot be transported intracellularly. Therefore, we screened fosfomycin resistant colonies by patching on M9 agar with glycerol phosphate as the sole carbon source. From resistant colonies collected on 256 μg/mL fosfomycin, *glpT* null colonies appeared at a rate of 1 per 100,000 total CFU, much lower than the frequency of the total resistant subpopulation at 1 per 300 total CFU (Supplementary Fig. 6c). While phenotypically null *glpT* mutants exist in cultures of Mu208, they comprised only a small fraction of the resistant subpopulation across most concentrations. However, when a strain only survived at a frequency of 1 per 10^5 total cells, because of genetic

mutation or fosfomycin concentration, the majority of those surviving colonies were phenotypically *glpT* null (Supplementary Fig. 6d). Thus, there was a rare subpopulation of resistant cells with null *glpT* function which exists at approximately 1 in 10^5 that is highly fosfomycin resistant. However, the vast majority of the resistant subpopulation has intact GlpT."

We have also added text in the Discussion to address this point, at line 500:

"Additionally, we did not expect to measure the fluorescence and survival of any *glpT* null mutants, as it is unlikely a cell which exists at 1 in 100,000 (Supplementary Figure 6c) would be visualized in an assay measuring 700-1000 cells. However, data presented here establishes that in Mu208, random, low frequency *glpT* inactivating mutants play a minor role in the resistant subpopulation. Following enrichment of the resistant subpopulation in broth, the population returned to baseline frequency when fosfomycin was removed, which confirmed that there was not a strong overall selection of stable resistant mutants (Figure 1c). Increasing expression of *glpT* by deleting *glpR*, which would not affect *glpT* null mutants, exerted a robust effect on the resistant subpopulation, consistent with *glpT* transcriptional regulation being a key driver of the resistant subpopulation (Figure 3b). When the subpopulation of wildtype Mu208 which survived at the breakpoint was assessed for *glpT* function, *glpT* null cells were determined to be present at a frequency of 1 in 10^5 (Supplementary Figure 6c). This rare subset of resistant cells only became dominant when sufficiently high concentrations of fosfomycin eliminated the vast majority of resistant cells with intact *glpT*. For wildtype Mu208, the resistant cell frequency dropped to 1 in 10^5 at 2048 µg/mL fosfomycin, and those surviving cells were mostly *glpT* null (Supplementary Figure 6d). This is consistent with the differing mechanisms of resistance; the vast majority of cells which survived fosfomycin had intact *glpT* but reduced expression. This population survived to 1024 µg/mL, and less so at 2048 µg/mL. *glpT* null cells likely had a higher single cell MIC due to complete *glpT* inactivation, and survived to at least 2048 µg/mL, but their very low frequency resulted in only a minor contribution to the composition of the total resistant subpopulation across the population."

8."Expression of the fosfomycin importer GlpT is heterogeneous and predicts survival in Fosfomycin". We know that the MIC values of different resistant subpopulation Are different. Are the expression levels of GlpT in these different subpopulation consistent? How about different clinical strains.

This is an excellent question that we are unable to answer at this time for Mu208. As for other clinical strains, we are limited in our ability to perform genetic manipulation and integrate a P_{glpT}*mcherry* reporter into the chromosome to assess this question.

We have added a discussion of this query at line 531:

"On the other hand, for any one growth condition, heterogeneity of resistance existed in wildtype Mu208 (Figure 6c-d). We attribute this heterogeneity partially to a broad distribution in P_{glpT} activity (Figure 2b), likely mediated by heterogeneity in CRP activity (Figure 3f). We observed susceptible and resistant cells, and the resistant subpopulation demonstrated a range of MICs; some cells survived at the breakpoint but not greater, while other resistant cells

survived beyond 2048 µg/mL. One might expect that the most resistant cells were those that had the absolute lowest level of *glpT* expression in the single-cell assays. However, the relative resistance within the subpopulation is likely the product of low *glpT* expression as well as FosA expression and activity, and *gshA*/glutathione synthesis. We observed that resistant cells demonstrated a broad range of *gshA* promoter activity, suggesting differences in glutathione abundance across the population (Supplementary Figure 8a). Thus, P_{glpT} activity is predictive of susceptibility, in the sense that if the cell demonstrates mCherry intensity >225, it will be susceptible (Figure 2d), but this cannot describe how resistant a single resistant cell is because of the important contributions of FosA and glutathione."

9"Transcriptional regulators GlpR and CRP control glpT expression and fosfomycin resistance"reveals the key regulatory axis of "glucose-CRP-glpT", providing an important mechanism for understanding how carbon sources affect antibiotic resistance. However, it has limitations in terms of physiological relevance, the complexity of the mechanism, the completeness of technical evidence, and the precise definition of resistant subpopulations. It is suggested that gel shift assays (EMSA) or chromatin immunoprecipitation (ChIP) could be used to verify the direct binding of CRP protein to the glpT promoter.

Thank you for this suggestion. We recognize that we have not fully demonstrated the biochemical interactions that govern these transcription factors. However, our data on the effects of GlpR and CRP on *glpT* are consistent with extensive, foundational literature from E. coli. Additionally, we observe high homology between GlpR and CRP of *E. coli* MG1655 and Mu208. CRP is 100% identical, right upper, while GlpR is 90% identical; right lower.

```
CLUSTAL O(1.2.4) multiple sequence alignment

gnl|ECOLI|PD00257     MVLGKPQTDPTLEWFLSHCHIHKYPSKSTLIHQGEKAETLYYIVKGSVAVLIKDEEGKEM     60
XOZ72703.1            MVLGKPQTDPTLEWFLSHCHIHKYPSKSTLIHQGEKAETLYYIVKGSVAVLIKDEEGKEM     60
                      ************************************************************

gnl|ECOLI|PD00257     ILSYLNQGDFIGELGLFEEGQERSAWVRAKTACEVAEISYKKFRQLIQVNPDILMRLSAQ     120
XOZ72703.1            ILSYLNQGDFIGELGLFEEGQERSAWVRAKTACEVAEISYKKFRQLIQVNPDILMRLSSQ     120
                      ************************************************************:*

gnl|ECOLI|PD00257     MARRLQVTSEKVGNLAFLDVTGRIAQTLLNLAKQPDAMTHPDGMQIKITRQEIGQIVGCS     180
XOZ72703.1            MARRLQVTSEKVGNLAFLDVTGRIAQTLLNLAKQPDAMTHPDGMQIKITRQEIGQIVGCS     180
                      ************************************************************

gnl|ECOLI|PD00257     RETVGRILKMLEDQNLISAHGKTIVVYGTR   210
XOZ72703.1            RETVGRILKMLEDQNLISAHGKTIVVYGTR   210
                      ********************************
```

Comparing the glpT promoter region and coding sequence (which includes GlpR operators), there is high conservation of the CRP and GlpR binding sites between E. coli MG1655 and Mu208 (DOI: 10.1046/j.1365-2958.1997.3651733.x), below.

```
CLUSTAL O(1.2.4) multiple sequence alignment

glpR          MKQTQRHNGIIELVKQQGYVSTEELVEHFSVSPQTIRRDLNELAEQNLILRHHGGAALPS    60
XOZ72748.1    MKQTQRHDAIIELVKKQGYVSTEELVEQFAVSPQTIRRDLNDLADQNRILRHHGGAALPS    60
              *******:.******:***********:*:***********:**:** ************

glpR          SSVNTPWHDRKATQTEEKERIARKVAEQIPNGSTLFIDIGTTPEAVAHALLNHSNLRIVT    120
XOZ72748.1    SSVNTSWHDRKATQTAEKERIARKVASQIPNGATLFIDIGTTPEAVAHALLDHENLRVVT    120
              ***** ********* **********.*****:******************:*.***:**

glpR          NNLNVANTLMVKEDFRIILAGGELRSRDGGIIGEATLDFISQFRLDFGILGISGIDSDGS    180
XOZ72748.1    NNLNVANTLMQKDDFRIILAGGELRSRDGGIIGEATLDFISQFRLDFGILGISGIDSDGS    180
              ********** *:************************************************

glpR          LLEFDYHEVRTKRAIIENSRHVMLVVDHSKFGRNAMVNMGSISMVDAVYTDAPPPVSVMQ    240
XOZ72748.1    LLEFDYHEVRTKRAIIENSRHVMLVVDHSKFGRNAMVNMGSISIVDAVYTDAMPPAGVMQ    240
              ********************************************:******** **..***

glpR          VLTDHHIQLELC    252
XOZ72748.1    VIKDNNLQLELC    252
              *:.*:::*****
```

We have edited the text at line 287 as follows

"In *E. coli*, *glpT* expression is regulated by the transcriptional repressor GlpR[27] and the carbon catabolite repression activator CRP[28] (cyclic-AMP receptor protein). We observed high amino acid homology between CRP of *E. coli* MG1655 and Mu208 (100% identity) as well as between GlpR from each strain (90% identity). We also identified putative binding sites in the *glpT*

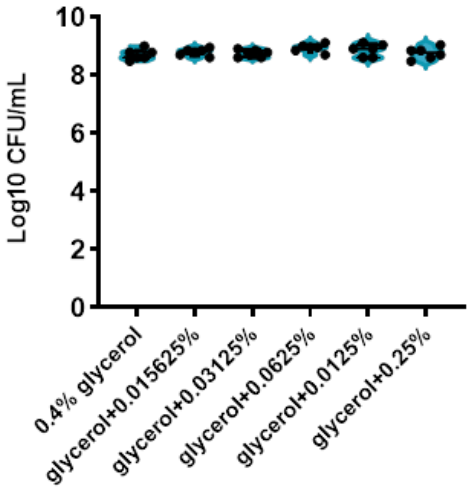
promoter for CRP and in the *glpT* coding sequence for GlpR based on high similarity to *E. coli*[27], suggesting a similar regulatory cascade in Mu208 (Figure 3a).”

10.“We measured glpT transcript abundance by qRT-PCR and found that excess glucose resulted in ~100-fold reduction in relative glpT transcript abundance (Figure 4b), confirming that glucose increases fosfomycin resistance via glpT repression.”Even though the expression level of glpT increased in the presence of glucose, this might be the main reason for the increase in drug resistance. Are there any other possible causes?

Thank you for this comment. Glucose would have many effects beyond decreasing *glpT* expression. However, our data support the conclusion that the major effect of glucose is glpT expression, based on Figure 4d. There, when *glpT* is constitutively expressed (P_{tet} promoter), so it cannot be regulated by GlpR or CRP, addition of glucose has no effect on the frequency of the resistant subpopulation, suggesting that if glucose has other effects beyond glpT, they are very minor. We have revised the text to clarify this point at line 544 in the discussion

"We conclude that the ability of environmental glucose to enhance the frequency of the resistant subpopulation (Figure 4) is largely due to repression of *glpT*. Glucose has global effects on cellular metabolism and could also enhance resistance by increasing glutathione synthesis, altering cell wall metabolism, or through other mechanisms. However, addition of glucose had no effect on the frequency of the resistant subpopulation in the strain engineered to have constitutive, non-regulated P_{glpT} activity (Figure 4d), demonstrating that any effects of glucose outside of affecting *glpT* transcription are minor."

11."When Mu208 was grown with glycerol as the sole carbon source, the frequency of the subpopulation resistant to 256 μg/mL fosfomycin was below 1%, but low concentrations of exogenous glucose resulted in a dose-dependent increase in the frequency of the resistant subpopulation (Figure 4c). Additionally, the ability of glucose to enhance the resistant subpopulation appeared to be common among 5 fosfomycin HR Enterobacter clinical isolates (Extended Data Figure 6). "Only under the condition of glycerol as the carbon source, the nutrients required for bacterial production are relatively less. Is this phenomenon caused by this reason? Figure 4 showed that as the glucose level increases, the number of bacteria also increases. Does the number of bacteria not continue to increase when the glucose concentration reaches a certain level?



Thank you for this comment. We have edited the text to clarify that *Enterobacter* grows robustly with glycerol as the sole carbon source and that there are no differences in the amount of bacteria in the cultures grown for Figure 4c. Adjacent are the raw data of CFU/mL of each condition from experiments presented in Figure 4c. We added the following at line 369:

"The effect of glucose was not the result of enhanced total growth of the cultures, as bacterial growth yield was equivalent in each condition."

12."Excess glucose in murine models of diabetes increases the frequency of the fosfomycin resistant subpopulation". The demonstration that this phenomenon occurs in two distinct murine models of diabetes (Type I and Type II) significantly strengthens the clinical relevance of the findings. It moves the mechanism from an in vitro observation to a potentially critical factor in treatment outcomes for a large patient population. Does the normal blood sugar concentration of the general population affect the number of drug-resistant subgroups?The in vivo correlation with hyperglycemia/diabetes is demonstrated in murine models. The translation of these findings to human infections, where the metabolic microenvironment may be more complex, requires clinical validation.

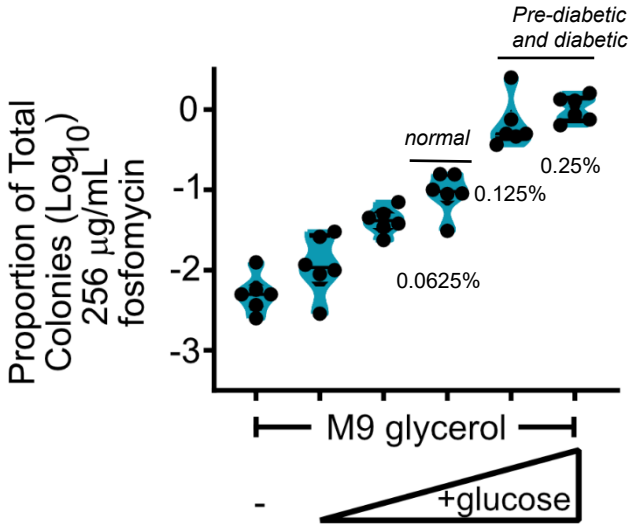
We thank the reviewer for this comment, and agree that much more work needs to be completed in the future to better understand how human glucose status and the microenvironment of the infection niche contribute to treatment outcomes. We sought in our manuscript to emphasize these findings are in mice, for example:

Line 51: "Due to the impact of environmental glucose on *glpT* expression, we observed that the resistant subpopulation was more frequent during infection in murine models of hyperglycemia/diabetes"

Line 105: “This is recapitulated in murine models of hyperglycemia/diabetes; the frequency of the fosfomycin resistant subpopulation is enhanced in the presence of excess glucose during infection.”
Line 429: “The fosfomycin resistant subpopulation is enriched in diabetic mice”
Line 451: “Figure 5. Glucose availability increases the frequency of the resistant subpopulation in murine models of diabetes/hyperglycemia”

We did observe an increase in the frequency of the resistant subpopulation between the infection input and the control mice (control mice not treated with streptozotocin or WT/*Lep*^{ob} controls) in Figure 5a,b, suggesting that even in non-diabetic mice there is an enrichment in the resistant subpopulation. The diabetic mice in our study have blood glucose levels of ~350 mg/dL, while non-diabetic humans have fasting blood glucose below 100 mg/dL. Converting to percent glucose (w/v), fosfomycin HR isolates would be exposed to <0.1% glucose in the blood and other body sites of normal patients but up to 0.35% glucose in diabetic patients. We have added labels to Figure 4c below to show where normal or diabetic blood sugar levels would be represented, along the *in vitro* glucose dose response. Of course, this is an oversimplification of the environment the bacteria encounter *in vivo*, but it provides some relative guidance, and we hope this study encourages future investigations using human clinical data.

c

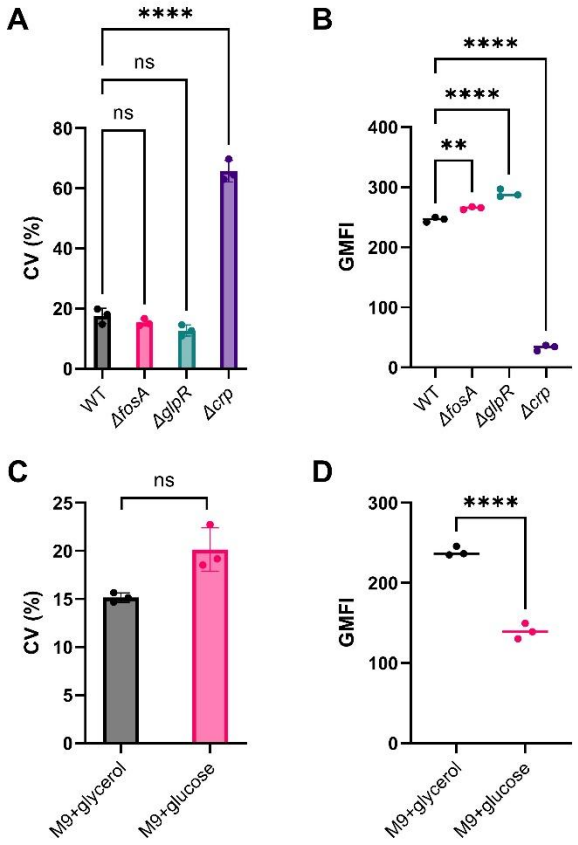


We have added text to the discussion to address this point at lines 550:

“We observed the effects of glucose *in vitro* and *in vivo,* and note that the concentrations used in M9 broth correlate well with levels of mammalian blood glucose. Non-diabetic blood glucose is below 100 mg/dL (0.1% glucose w/v) when fasted or below 200 mg/dL (0.2% w/v) non-fasted, and diabetic blood glucose concentrations up to and exceeding 240 mg/dL (0.24% w/v) are observed in humans[40] and 350 mg/dL (0.35%) in the mice used in this study[41,42]. We observed more than a 1-log increase in the fosfomycin resistant subpopulation frequency with addition of as little as 0.0625% glucose (Figure 4c). Together, these data indicate that host-derived glucose could have meaningful effects on fosfomycin heteroresistance at concentrations relevant to non-diabetic and diabetic physiology.”

13. The single-cell data in Figures 2, 3, and 4 are compelling. Could the authors provide a quantitative measure of the heterogeneity (e.g., coefficient of variation) for PglpTmCherry expression under different conditions (e.g., +/- glucose) to supplement the frequency plots? This would further strengthen the description of the population shift.

Thank you for your suggestion. We quantified single-cell heterogeneity of P*glpT*-mCherry expression by calculating the coefficient of variation (CV = SD/mean), the extent to which the distribution of individual cells varies from the mean, for each biological replicate prior to fosfomycin exposure. The CVs of P*glpT*-mCherry expression only show a slight decrease in the Δ*fosA* and Δ*glpR* strains compared to WT (no significant differences), with a significant increase in Δ*crp* relative to WT (panel A). However, this change is largely driven by the very low fluorescence signal in the Δ*crp* strain. We quantified geometric mean fluorescence intensity (GMFI) among these strains, panel B. While GMFI does not measure heterogeneity, it is informative of the shift in different strains/conditions. Additionally, growth in glucose, which strongly represses *glpT*, resulted in a modest increase in CV relative to glycerol with a significant reduction in GMFI (panels C-D).
We also note that the percent resistance indicated in each fluorescence panel is equivalent to area under the curve calculation for the resistant cells, which further reinforces the differences in the strains/conditions following fosfomycin exposure.



14. Extended Data Figure 6 nicely shows the glucose effect in other clinical isolates. A brief comment in the main text or discussion emphasizing that this mechanism is not unique to Mu208 would be helpful to underscore its broader relevance.

Thank you. As described for Major comment #1 above, we have re-organized our results section to integrate our data from other isolates.

Reviewer #3 (Remarks to the Author):
Choby et al. studied fosfomycin heteroresistance in one E. cloacae complex isolate and one susceptible control. The authors investigated the interplay of GlpT, fosA, and glucose on fosfomycin heteroresistance and tested the effects of host glucose levels on heteroresistance in murine models of infection with hyperglycemia. The manuscript is well written, with a clear language. Many supporting details are provided in the supplementary information. The manuscript figures are sufficiently clear, but the language used in the axis labels can be improved for clarity.

We thank the reviewer for these positive comments.

Minor comments:
105: What species within E. cloacae complex is Mu208? WGS data can be used for genomic species determination, e.g. with GTDBtk.

Mu208 is *Enterobacter kobei*, as confirmed by Average Nucleotide Identity (ANI) performed as part of Genbank submission in NCBI https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_048016555.1/#:~:text=Mu208%20%2D%20Genome%20%2D%20Assembly%20%2D%20NCBI.

We have updated the text in line 114: "We assessed the features of fosfomycin heteroresistance (HR) in a clinical isolate of the *Enterobacter cloacae* complex, *E. kobei* strain Mu208."

Across several figures: Be consistent when indicating percentages and proportions in axis labels, e.g. 'Resistant to fosfomycin (%)' vs. '% resistant to fosfomycin'. Could you improve labels such as 'Frequency of population (%)' e.g. to 'Share of population (%)'?

Thank you for the feedback. We have updated the figures as recommended. All data from microscopy are now labeled as "Share of population (%)". For data with percent resistant, it has been modified to say "Population resistant to 256 µg/mL fosfomycin (%)".

493: How was the reference genome of Mu208 assembled?

We thank the reviewer for identifying this omission. We have added the following details at line 626:

"Porechop[34] was used to trim residual adapter sequence from the Oxford Nanopore Technology (ONT) reads that may have been missed during basecalling and demultiplexing. De novo genome assemblies were generated from the ONT read data with Flye[35] under the nano-hq (ONT high-quality reads) model. Additional Flye options initiate the assembly by first using reads longer than an estimated N50 based on a genome size of 6Mbp. Subsequent polishing used the Illumina read data with Pilon[36] under default parameters. To reduce erroneous assembly artifacts caused by low quality nanopore reads, long read contigs with an average short read coverage of 15x or less were removed from the assembly. Assembled contigs were evaluated for circularization via circlator[37] using the ONT long reads. Assembly annotation was then performed with Bakta[38] using the Bakta v5 database."

724: define MHA
We have defined, now at line 1103 of the Supplement "Mueller Hinton agar (MHA)"

Add description for supplemental video

We have added into the Supplementary materials: "Supplementary Video. Mu208 *att*:: P_{glpT}*mCherry* time lapse microscopy immediately before and for 5 h following addition of 128 µg/mL fosfomycin. Time is shown in the upper left and scale bar in the bottom right."

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):
The revised manuscript addressed most of the initial concerns either by additional experimentation or by highlighting the challenges that may hinder addressing some of the concerns. The following remain unaddressed or not fully addressed (numbered in relation to the original major concerns):

Major concern 6: The link to host metabolic state is among the most interesting and possibly clinically relevant findings of this study (which is also likely the reason this is referred to in the title); hence, it would require more robust proof. I thank the authors for clarifying the limitations/challenges. However, as it stands, there is not enough/robust evidence that an infection in the diabetes/hyperglycemina model would be worse than in a standard infection in non-diabetic mice. If the survival assays (similar to those in Fig 1e) for these diabetic models show even subtle survival shifts, these would be complementary to the shown CFU differences (which are relatively subtle themselves), further supporting the claims. An explicit mention of the limitations/challenges to demonstrate clearer link between the metabolic state and the treatment outcome due to these HR strains should be included. Alternatively, the authors could test another strain/species other than Mu208 in these assays. For example, is there a K. pneumoniae or other from those tested in Supp Fig 12 that may be more suitable to demonstrate the effect of host glucose (host disease state)?

We thank the reviewer for the feedback on this revision. We emphasize metabolic environment in the title and elsewhere largely because this is the first known report of carbon metabolism controlling antibiotic heteroresistance, which expands our mechanistic understanding of phenotypic heterogeneity in the context of antibiotic resistance. We specifically avoided including "host metabolic environment" in the title. Further, we do not contend that infection in the diabetes model would be worse, due to the limitations discussed in our first response. Rather, we are careful to conclude that "These data suggest that during infection, Mu208 responds to host glucose and is more resistant to fosfomycin. More broadly, these results show how the host metabolic environment encountered by pathogens could impact antibiotic resistance." and "Most surprisingly, our data suggest that the host metabolic environment or co-morbidities may affect the outcome of antibiotic treatment. We observed that excess host glucose in models of hyperglycemia/diabetes increased the frequency of the resistant subpopulation in strain Mu208 during infection."

We have added the following sentence to the final paragraph of the results "Because fosfomycin failed to treat wildtype mice infected with Mu208 (Figure 1e,i), we were unable to compare the outcomes of infection and fosfomycin treatment of STZ-treated or *Lep^{ob}/Lep^{ob}* mice relative to wildtype mice."

Major concern 1: The edits and additional discussion mostly addressed major concern 1. However, a more explicit limitation statement (that the observed results could be due to the high level of resistance rather than HR per se) should be included in the main text and not only in the response to reviewers. Further, in the discussion added at line 463: “clinically relevant resistant subpopulation can exist as low as 0.0001% of the population” How was that assessed? There is no citation associated with that statement. This should be clarified and/or supported with a citation. Also, Line 470, “a population survive” could be possibly revised to “a population that survives”?

We thank the reviewer for these clarifying points.

Statement on limitation: We have added the following to the first paragraph of the discussion: “Our conclusions regarding the ability of Mu208 to cause treatment failure are limited because of its high frequency resistant subpopulation, making it challenging to separate the effects of heteroresistance versus overall population resistance.”

Line 463: We appreciate this point and should have been more specific. Originally, we were referring to the field’s definition of heteroresistance, which extends to 0.0001-0.00001%. Instead, we have revised this sentence as follows: “which is relatively high considering that isolates with resistant subpopulations as low as 0.001% of the population caused treatment failure in mice[8,32]”.

Line 470: corrected as suggested

Reviewer #2 (Remarks to the Author):
The author has already addressed all the issues; it is recommended for acceptance.

We thank the reviewer for their service and feedback.