nature portfolio

Peer Review File

Methionine-driven methylation modification overcomes plasmid-mediated high-level tigecycline resistance

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Please refer to the "Review Attachments".

Reviewer #2 (Remarks to the Author):

This manuscript elaborated the metabolic profiles between tet(X)-negative and -positive bacteria, uncovering that exogenous supplement of Met can effectively restore the susceptibility of tet(X)-carrying pathogens to tigacycline. The efficacy of this combination therapy was evidenced in multiple animal infection models in vivo. Mechanistically, the combination of Met and Tig could enhance bacterial PMF, which subsequently promotes intracellular accumulation of Tig. Meanwhile, exogenous Met activates the Met pathway, promotes the production from Met to methyl donor SAM, especially enhances the 5mC methylation modification in the promotor region of the tet(X4) gene. The increase in drug accumulation and the decrease in tet(X) gene expression collectively contribute to cell death. The manuscript is overall well prepared, however, there are several inconsistencies in the results of the mechanism analysis and some other issues listed below need to be carefully considered.

1. Figure 1K: it is shown that sensitive bacteria exposed to tigecycline exhibit almost zero ATP levels, indicating that the bacteria may have died. This could amplify the differences in mRNA expression levels between sensitive and resistant bacteria. Therefore, I suspect that using an antibiotic treatment concentration of 1*MIC for sensitive bacteria in this experiment is excessive, and a subinhibitory concentration may be more appropriate.
2. Line 229: expression levels of dcm and mtn were increased by exogenous Met, but metK were reduced (Fig. 3G); Lin236: protein production of MetK, Dcm were increased; Why the expression of dcm and mtn genes increased and metK expression decreased; but the protein production of MetK and DCM were increased. This is the first point of inconsistency.
3. Figure 4E: under arabinose induction, the production of metK significantly decreased and Dcm significantly increased after the addition of Tig and Met, which was inconsistent with the conclusion that both MetK and Dcm increased in line 236 of the manuscript. This is the second point of inconsistency.
4. Figure 4 FGH: the legend is inconsistent with the annotation below it. This is the third point of inconsistency.
5. Lines 136-161: the results of the second part show that the basic mechanism of Tig-R and Tig-S exhibiting two different energy demand patterns is mainly caused by NUO-mediated NADH dehydrogenase. The above results do not have a strong logical relationship with the following results; therefore, I suggest that the authors rearrange this part of the results.
6. Figure 2D-2E: the authors selected several strains of clinical Tig resistant bacteria, why not select several strains of clinical Tig sensitive bacteria as well for conducting the assays?
7. Line 215: PMF has two components, $\Delta\Psi$ and ΔpH, therefore why measure $\Delta\Psi$ only and not ΔpH? The measure of ΔpH should be added. When testing $\Delta\Psi$, positive control should be added. In addition, the authors mentioned that exogenous methionine (Met) can enhance the uptake of tigecycline by upregulating the ΔpH component of the bacterial proton motive force (PMF). Could the authors discuss

the reasons why Met facilitates the uptake of tigecycline but does not exhibit a synergistic effect with it?
8. Why did E. coli J53 be selected instead of E. coli B3-1-(tet(X4)) or E. coli DH5α- (pUC19-tet(X4)), which had been phenotypically validated before, when the knockout bacteria were selected for testing?
9. When testing Minimum inhibitory concentration (MIC), quality control strains need to be added.
10. Some bar graphs are presented in an inverted format, while others are in a standard format. Is there a specific reason for this variation? A uniform presentation of the bar graphs might improve readability for the readers.

Reviewer #3 (Remarks to the Author):

• What are the noteworthy results?
The work by Fang and collaborators highlights the importance of epigenetic regulation in bacterial metabolism and establishes the mechanisms underlying regulation through the methionine-mediated TetX regulon, which enhances the efficacy of the antibiotic tigecycline. Together, the findings presented in this article underscore bacterial biochemistry and metabolic homeostasis between tigecycline-sensitive and resistant strains, through a series of well-designed metabolomic and transcriptomic experiments. Additionally, the article addresses the regulation of tetX gene expression. The results presented in this article demonstrate a novel strategy in which methionine has great potential as an adjuvant targeting infections caused by multidrug-resistant bacteria containing the tet(X) resistance-associated gene.
• Will the work be of significance to the field and related fields? How does it
This work is definitely significant, as Fang and collaborators provide evidence for reversing resistance in tetX+ strains through methionine supplementation, creating an opportunity for research in developing adjuvants from metabolites that enhance the effect of antibiotics.
• compare to the established literature? If the work is not original, please provide relevant references.
Even though components that enhance the bactericidal activity of tigecycline have been described, the mechanisms associated with this effect are not well understood. In this article by Fang and collaborators, the authors delineated the specific metabolic pathways involved that can be used to enhance the efficacy of an antibiotic , highlighting a close relationship between metabolic homeostasis and the final fate of the bacterial cell.
• Does the work support the conclusions and claims, or is additional evidence needed?
Throughout the article, the work presents clear evidence for its claims and conclusions. The experimental design reflects the complexity and relevance of this study. However, to provide the reader with greater understanding, we suggest the following:
• Expand on the rationale for choosing a Klebsiella pneumoniae strain to introduce the tetX4 constructs.
• In the methodological description of the transcriptome that references bibliography 42 of the article, the cut-off used for the fold change is 2. Why is a lower cut-off used for this work?
• Regarding Fig. 3, panels M and N, what explanation can be inferred if, in the presence of methionine and tigecycline, the transcripts of the tetX4 gene seem to disappear but can still be detected at the protein level under the same conditions (methionine and tigecycline present)?
• At what time points was relative expression determined in Fig. 3, panel G? Were the 6 hours maintained?

• The authors describe an acute lung infection model in mice infected with K. pneumoniae 585-1 positive for tet(X4). However, a concern regarding this model lies in the intraperitoneal infection route with K. pneumoniae and its subsequent evaluation of lung damage caused 6 hours post-infection. This result does not represent a validated model for studying lung damage through intraperitoneal inoculation. I suggest establishing a representative model of acute lung damage to better approach the infectious process caused by K. pneumoniae.
Minor revisions:
• The quality of the legends in Fig. 1 is poor, particularly panels D and E, which are not clearly visible.
• In Fig. 2, panel A, the legends are misaligned.
• In Fig. 3, panel N, the legend of the detected protein is missing.
• The quality of the legends in Fig. 4 is poor, particularly panels A, B, C, J, and K.
• Line 34: Mention which infection models in animals were used. Lines 650-651: Correct "K pneumonias" to "K. pneumoniae". Line 651: Does the infection route refer to intraperitoneal? Please specify.
Are there any flaws in the data analysis, interpretation, and conclusions? Do these prohibit publication or require revision?
Is the methodology sound? Does the work meet the expected standards in your field? Yes, the methodology is solid, and the experiments are complementary, allowing the reader to resolve most doubts. However, it is necessary to use a representative model of acute lung infection with K. pneumoniae to evaluate the adjuvant activity of methionine using the antibiotic tigecycline.
Is there enough detail provided in the methods for the work to be reproduced? Yes, the experiments are described in detail, and when the text does not explicitly state the details, a reference is provided for the specifications, as in the case of the construction of puc19.

Reviewer #4 (Remarks to the Author):

"I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts."

Reviewer #5 (Remarks to the Author):

The manuscript reports on a surprising discovery that resistance against the antibiotic tigecycline is mitigated through additional supply of methionine. The authors report several intereeting data on the physiology of methionine treated and untreated cells and show that met serves as sensitizer of tigecycline susceptibility. This is in line with recent findings of methionine increasing general susceptibility against macrolide(!) antibiotics in Streptococcus suis (PMID: 38230928)

The observation is very interesting and the authors provide compelling data for the direct link between methionine and increased susceptibility. However, there are some concerns in respect to the actual mechanisms and whether it is specific (as proposed by the authors and in the title) or a more general,

multi-layered mechanisms of methionine (in line with PMID: 38230928).

(1) methylation mechanism:

Since pUC19 ius a high copy vector, I am particularly interested by the question, how the Met effect plays out in another, low copy vector. My feeling would be that this effect should be less pronounced, as methylation does not become a limiting factor anymore in a low copy plasmid. At a broader level, I am a generally puzzled that a vector of ~4-8 kb size (even if present at high copy, i.e, ~20 copies) would make a difference. This makes maybe ~100-200 kb extra DNA, which is less than 10% of the total genome (E coli. 3,400 kb!) that needs to be extry methylated. This less than 10% of total DNA and should not make a difference. How does the complete methylation pattern of E. coli actually look like under these conditions? Moreover, would the effect be completely eradicated in a vector that does not carry dcm motifs?

(2) alternative hypotheses:

As much, as I like the idea of Met being important for dcm methylation, methionine could also work at other levels. What about the recent discovery that methionine works through decreasing efflux pump expression and general ROS stress sensitizer in Streptomyces reported by other authors recently (PMID: 38230928)? Could supply of methionine cause similar unspecific effects and increase ROS effects coming from unfruitful H2O2 production from tetX4, etc.? I don't think that the dcm expression/titrating experiment provides suffcient evidence that it is specific dcm patterns that cause the effect (it could be simply more SAM available of other biochemistry, etc.).

In summary, the data presented in the manuscript do not show that dcm 5mC methylation PRECISELY modulates tet(X) resistance expression. This is all interesting data, but not bolstered by any mechanistic explanation or sufficient controls/experiments by the authors. The authors are well-adviced to not over interpret their data (and overstate their findings, e.g. in the title/abstract). Especially, since the effect of Met in vivo is probably multi-layered - and eventually not specific (see PMID: 38230928), I believe that this needs to be reflected in the abstract and throughout the manuscript.

In this manuscript, the authors discovered the potentiation of L-methionine (Met) to tigecycline (Tig) in treatment of tet(X)-positive bacterial infections. Using detailed differential metabolic and flux analysis, they found the significant downregulated cysteine and Met metabolism pathway in tet(X)-positive bacteria and demonstrated the synergistic mechanisms of using exogenous Met to re-sensitize tet(X)-positive pathogens to Tig: both promotes intracellular Tig accumulation and enhances 5mC methylation. They not only showed success of using Met + Tig to eliminate tet(X)-positive E. coli in vitro, but also demonstrated effectiveness of such treatment in animal tests. Their work can not only find direct applications in antibiotic treatment of multi-drug resistant pathogens, but also provides a new paradigm to potentiate antibiotic efficacy through harnessing cellular metabolic networks and epigenetic modifications. I would recommend this manuscript to "Nature Communications" after minor revisions.

1. Figure 2B, + Met test similar to Figure 2A should also be added as a control. In Figure 2C, I would recommend (optional) using "finer" time intervals to investigate impact of adding Met before or after the addition of Tig, such dynamics of metabolic reprogramming might be interested to the readers.

2. In Figure 2F, it seems that the initially inhibited bacteria resume growth after 6 hours. Could the authors explain or discuss this? Furthermore, will continuous supplying Met, Tig, or both keep the inhibition effect? Also, its title should not be named "survival".

3. In Figure 2G, the change of +Tig+Met does not well correspond to the change of growth curve in Figure 2F, can the authors explain this or showed the detailed calculation/data processing?

4. In Figure 3B, all (CCCP, Tig, Met) + should be added. In Figure 3C, error bar for +Tig+Met should be added.

5. Will the pathogens invade the animal cells? If so, I would also recommend (optional) measure intracellular Met or Tig (inside animal cells) to discuss/evaluate the effectiveness of using +Tig+Met with in vivo treatment. Furthermore, the authors should also discuss such metabolic reprogramming effects in related animal cellular pathways.

6. Page 4, line 73, Fe iron → either Fe or iron

7. Page 11, line 289~292, the authors described the Dcm as "only slightly inhibited" in Figure 4F and "significantly inhibited" in Figure 4G, but using the same color (blue) labeling, please either correct the Figure or the description.

Reviewer #1

In this manuscript, the authors discovered the potentiation of L-methionine (Met) to tigecycline (Tig) in treatment of tet(X)-positive bacterial infections. Using detailed differential metabolic and flux analysis, they found the significant downregulated cysteine and Met metabolism pathway in tet(X)-positive bacteria and demonstrated the synergistic mechanisms of using exogenous Met to re-sensitize tet(X)-positive pathogens to Tig: both promotes intracellular Tig accumulation and enhances 5mC methylation. They not only showed success of using Met + Tig to eliminate tet(X)-positive E. coli in vitro, but also demonstrated effectiveness of such treatment in animal tests. Their work can not only find direct applications in antibiotic treatment of multi-drug resistant pathogens, but also provides a new paradigm to potentiate antibiotic efficacy through harnessing cellular metabolic networks and epigenetic modifications. I would recommend this manuscript to "Nature Communications" after minor revisions.

Response: Thanks for your insightful comments for improving our manuscript. We have revised our manuscript thoroughly based on your comments. All changes are highlighted by red in the revised manuscript.

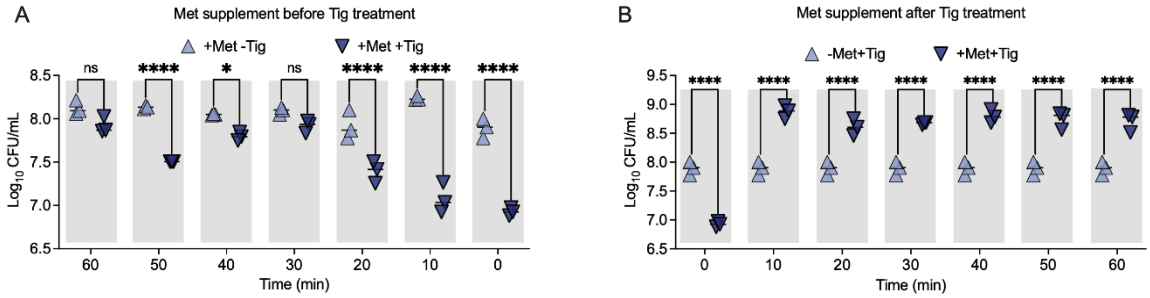
1. Figure 2B, + Met test similar to Figure 2A should also be added as a control. In Figure 2C, I would recommend (optional) using "finer" time intervals to investigate impact of adding Met before or after the addition of Tig, such dynamics of metabolic reprogramming might be interested to the readers.

Response: Thanks for your comments. In Figure 3B, Met alone has been added as a control. Meanwhile, "finer" time intervals (10 min) have been supplemented to investigate impact of adding Met before or after the addition of Tig. Interestingly, we found that only Met was added previously within 20 min or added with Tig at the same time, the re-sensitization effect of combination treatment (Met and Tig) could be observed **(Fig. S8)**, which further supported

our hypothesis that, metabolic reprogramming happened immediately or at least during one replication cycle to disturb the resistance procedures in which bacteria respond to antibiotic stress, rather than modulating those procedures that pre- or post- antibiotic exposure.

To highlight this point, we have revised our manuscript as follows.

Interestingly, this action was only observed when Met and Tig were added simultaneously or pre-treated within 20 min, not applied for the condition that Met was added before or after Tig exposure over 1 h. ***(Fig. 3C and S8)***. **(Line 176-178)**



Supplementary Fig. 8. Effect of adding Met before or after Tig treatment in E. coli B3-1 (tet(X4)).

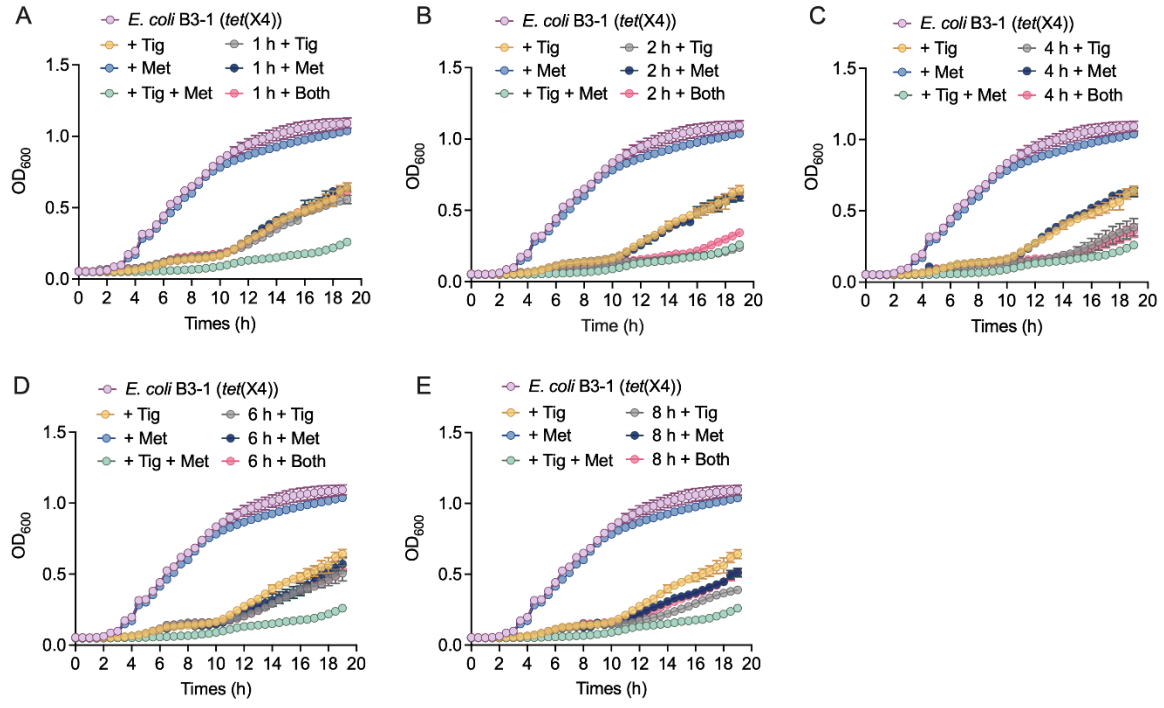
(A) *The impact of adding Met at 60 min, 50 min, 40 min, 30 min, 20 min, 10 min before Tig treatment to E. coli B3-1 (tet(X4)).* ***(B)*** *The impact of adding Met at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min after Tig treatment to E. coli B3-1 (tet(X4)). Data were displayed as mean ± SEM, and statistical significance was determined by unpaired t-test. *P < 0.5, ****P < 0.0001, ns, not significant.*

2. In Figure 2F, it seems that the initially inhibited bacteria resume growth after 6 hours. Could the authors explain or discuss this? Furthermore, will continuous supplying Met, Tig, or both keep the inhibition effect? Also, its title should not be named “survival”.

Response: Thanks for your comments. **Fig. 3F (former Fig. 2F)** displayed the growth curves of *E. coli* B3-1 (*tet*(X4)) under different condition within 12 h, we postulated that since cells was diluted 1:1000 to fresh LB broth at the beginning, it is likely that Tig and Met were almost depleted after 8 h and then the remaining bacteria grow again. Moreover, we have assessed the impact of continuous supplying Met, Tig, or both, and it was found that continuous interval supplying drugs had slight effect on sensitization **(Fig. S9)**. We have deleted the title accordingly.

To highlight this point, we have revised our manuscript as follows.

*Moreover, continuous supplementation with Met, Tig or both at intervals of 1, 2, 4, 6, 8 h after combination therapy had slight effect on sensitization **(Fig. S9)***. **(Line 178-180)**



Supplementary Fig. 9. Effect of continuous supplementation on the Tig plus Met treatment.

Growth curves of E. coli B3-1 (tet(X4)) within 19 h in the presence of Tig (32 μg/mL), Met (20 mM) or both, as well as continuous adding Met, Tig or both at 1 h ***(A)****, 2 h* ***(B)****, 4 h* ***(C)****, 6 h* ***(D)*** *and 8 h* ***(E)*** *after combination treatment.*

3. In Figure 2G, the change of +Tig+Met does not well correspond to the change of growth curve in Figure 2F, can the authors explain this or showed the detailed calculation/data processing?

Response: Thanks for your comments. In **Figure 3F (former Figure 2F)**, overnight cultures were diluted 1:1000 in 1 mL fresh LB broth and challenged Tig treatment or Met treatment or both of them, then bacterial suspensions cultured in a clear UV-sterilized 96-well microliter plate with total volume of 200 μL. In **Figure 3G (former Figure 2G)**, *E. coli* B3-1(*tet*(X4)) was cultured overnight, and then 1:100 diluted to fresh LB broth for 4 h to log phase, then bacterial cells were centrifuged at 4000 rpm for 7 min and the cells were resuspended in M9 minimal medium arrive at OD_{600} of 0.1, and antibiotics or metabolites were added at given concentrations, we performed bacterial enumeration at 6 and 12 h. Overall, in these two experiments, the initial bacterial amount, bacteria status and culture medium were totally different, and even the culture system and oxygen accessibility were different, thus there were different growth characteristics, characterizing the bacteriostatic and bactericidal activity of +Tig+Met, respectively. The experimental details have been supplemented in the revised manuscript.

4. In Figure 3B, all (CCCP, Tig, Met) + should be added. In Figure 3C, error bar for +Tig+Met should be added.

Response: Thanks for your comments. We have added "+" in **Figure 4B**, as well as error bar for +Tig+Met in **Figure 4C**.

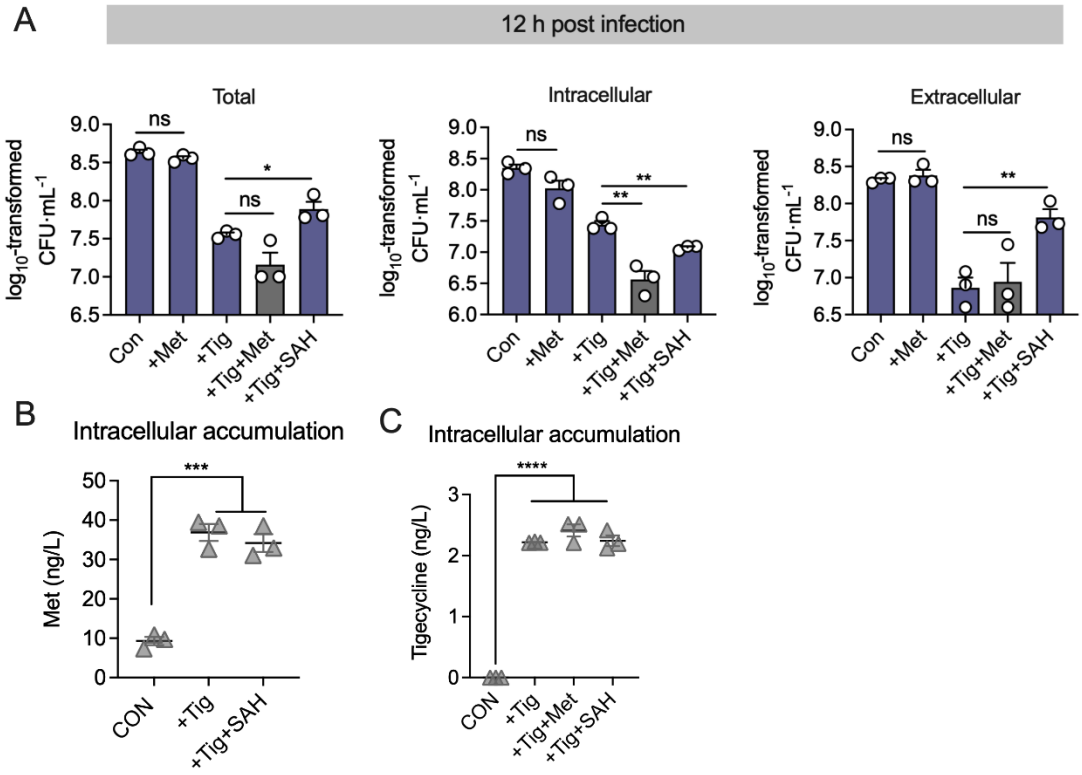
5. Will the pathogens invade the animal cells? If so, I would also recommend (optional) measure intracellular Met or Tig (inside animal cells) to discuss/evaluate the effectiveness of using +Tig+Met with *in vivo* treatment. Furthermore, the authors should also discuss such metabolic reprogramming effects in related animal cellular pathways.

Response: Thanks for your comments. We have supplemented related experiments to assess the elimination efficacy of +Tig+Met against intracellular bacteria using RAW264.7 cells model. As shown in **Fig. S19**, we found that the combination of Met and Tig significantly reduced intracellular bacterial loads, and this process was accompanied by the increase of Met accumulation in RAW264.7 cells. In addition, we also discuss such metabolic reprogramming effects on cellular pathways in the Discussion section.

To highlight this point, we have revised our manuscript as follows.

In E. coli B3-1 (tet(X4))-infected macrophages RAW264.7 cells, we found that the combination of Met and Tig significantly reduced the loads of intracellular bacteria, and this process was accompanied by an increased intracellular accumulation of Met ***(Fig. S19)*** **(Line 309-312)**

In fact, Met is also complicated in T cell activation and differentiation, such as manipulating epigenetic reprogramming in CD4($^{+}$) T helper (Th) cells. Furthermore, sufficient Met is critical for suppressing Th17 cell proliferation and the subsequent cytokines production, and the underlying mechanism also involves methylation modification (increased histone H3K4 methylation). Therefore, we speculated that alleviated inflammatory response in Tig plus Met-treated mice can be explained by not limited to the efficacy of combination therapy but also Met-mediated immunotherapy. **(Line 404-410)**



Supplementary Fig. 19. Combined treatment eliminates intracellular bacteria in RAW264.7 cells.

(A) *Bacterial loads of E. coli B3-1 (tet(X4)) at 12 h after co-incubation with RAW264.7 cells in the presence of Met, Tig, Tig + Met or Tig + SAH. SAH was used as a methyltransferase inhibitor. Tig (16 μg/mL), Met (20 mM), SAH (10 mM)* ***(B)*** *The intracellular Met levels of RAW264.7 cells at 12 h post infection.* ***(C)*** *The intracellular Tig levels of RAW264.7 cells at 12 h post infection. Data were displayed as mean ± SEM, and statistical significance was determined by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

6. Page 4, line 73, Fe iron → either Fe or iron.

Response: Thanks for your comments. We have corrected it accordingly.

7. Page 11, line 289~292, the authors described the Dcm as “only slightly inhibited” in Figure 4F and “significantly inhibited” in Figure 4G, but using the same color (blue) labeling, please either correct the Figure or the description.

Response: Thanks for your comments. We have corrected it in **Fig. 5G (former Fig. 4G)**, which is consistent with the results in **Fig. 5E (former Fig. 4E)**.

Reviewer #2

This manuscript elaborated the metabolic profiles between tet(X)-negative and -positive bacteria, uncovering that exogenous supplement of Met can effectively restore the susceptibility of tet(X)-carrying pathogens to tigacycline. The efficacy of this combination therapy was evidenced in multiple animal infection models in vivo. Mechanistically, the combination of Met and Tig could enhance bacterial PMF, which subsequently promotes intracellular accumulation of Tig. Meanwhile, exogenous Met activates the Met pathway, promotes the production from Met to methyl donor SAM, especially enhances the 5mC methylation modification in the promotor region of the tet(X4) gene. The increase in drug accumulation and the decrease in tet(X) gene expression collectively contribute to cell death. The manuscript is overall well prepared, however, there are several inconsistencies in the results of the mechanism analysis and some other issues listed below need to be carefully considered.

Response: Thanks for your insightful comments for improving our manuscript. We have revised our manuscript thoroughly based on your comments. All changes are highlighted by red in the revised manuscript.

1. Figure 1K: it is shown that sensitive bacteria exposed to tigecycline exhibit almost zero ATP levels, indicating that the bacteria may have died. This could amplify the differences in mRNA expression levels between sensitive and resistant bacteria. Therefore, I suspect that using an antibiotic treatment concentration of 1*MIC for sensitive bacteria in this experiment is excessive, and a subinhibitory concentration may be more appropriate.

Response: Thanks for your comments. We have added 1/4*MIC or 1/2*MIC concentration of tigecycline to both S and R suspensions to fully assess the difference of energy demands patterns between Tig-S and -R. The ratio of ATP/OD_{600} was used to normalize the initial

differed cells amount. As shown in **Fig. 2C**, ATP demand rapidly increased in R but decreased in S under subinhibitory concentration to MIC of Tig.

To highlight this point, we have revised our manuscript as follows.

*As shown in **Fig. 2C**, the ATP demand rapidly increased in R but decreased in S under subinhibitory concentrations of Tig.* **(Line 138-140)**

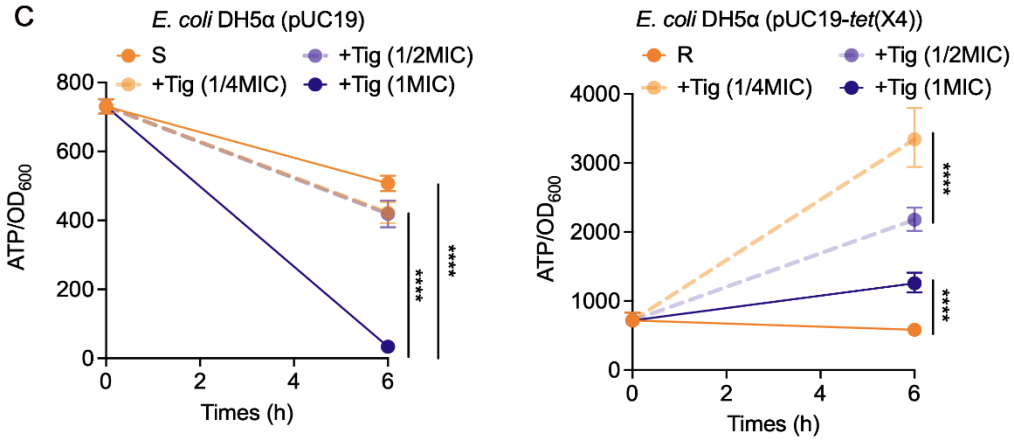


Fig. 2. Differential energy demand patterns between tigecycline-resistant and -susceptible bacteria under antibiotic exposure.

***(C)** Ratio of the intracellular ATP and OD_{600} during growth of E. coli DH5α (pUC19) (S) and E. coli DH5α (pUC19-tet(X4)) (R) under different Tig concentrations at 0 h and 6 h.*

2. Line 229: expression levels of dcm and mtn were increased by exogenous Met, but metK were reduced (Fig. 3G); Lin236: protein production of MetK, Dcm were increased; Why the expression of dcm and mtn genes increased and metK expression decreased; but the protein production of MetK and DCM were increased. This is the first point of inconsistency.

Response: Thanks for your comments. We performed three independent replicates of these experiments and obtained similar results. The possible mechanisms explaining why mRNA expression of genes decreased but protein expression increased include: (1) Translation is extremely efficient, even though mRNA were at the low level; (2) The protein degradation mechanism is inhibited, leading to protein accumulation; (3) There may be a large amount of

stored proteins or reused events within the bacterial cells; (4) There may be some essential post-translational modifications that enhance the stability and function of the protein, etc.

For *metK* gene, an example reported that manipulating the phosphate and nitrogen response factors could regulate *metK* gene expression in Gram-positive *Saccharopolyspora erythraea* (*Microb Cell Fact*, **2022**, 21, 120), which provided a rationale to regulate the *metK* gene expression at the transcriptional level in prokaryotes. Collectively, we supposed that, L-Met supplementation increased metK protein utilization, which in turn exacerbated the depletion of the L-met pool, where there may be a negative feedback mechanism that regulates the *do novo* transcriptional expression of *metK* gene, and thus reaching to a balance between the substrate L-met and the downstream product SAM.

3. Figure 4E: under arabinose induction, the production of metK significantly decreased and Dcm significantly increased after the addition of Tig and Met, which was inconsistent with the conclusion that both MetK and Dcm increased in line 236 of the manuscript. This is the second point of inconsistency.

Response: Thanks for your comments. We monitored the Met metabolic flux both in both clinical strain *E. coli* B3-1(*tet*(X4)) **(Fig. 4I)** and engineered *E. coli* MG1655 (pBAD-*tet*(X4)) **(Fig. 5E-5H)**. The changes of Met metabolic flux caused by the addition of both (Tig and Met) in these two strains were not completely consistent, but considering the precision and complexity of biological expression system, especially the different concentrations of arabinose dominantly regulated the *tet*(X4) expression and 5mC methylation would not become the only regulatory factors in *E. coli* MG1655 (pBAD-*tet*(X4)), thus we reckoned that these inconsistencies were reasonable. Most importantly, the changes of key metabolites and enzymes in 5mC methylation process were consistent (Dcm, SAM and SAH).

4. Figure 4 FGH: the legend is inconsistent with the annotation below it. This is the third point of inconsistency.

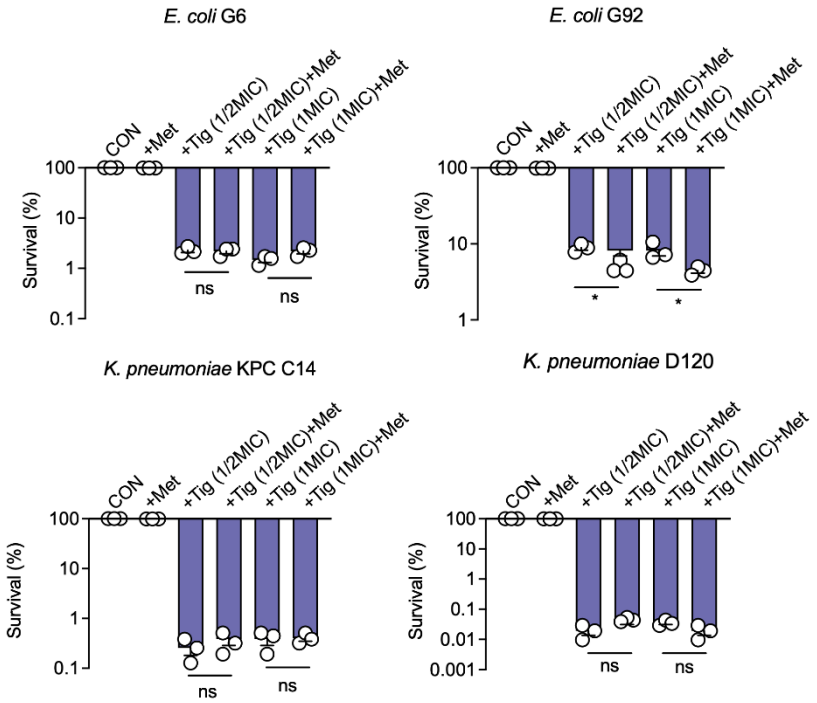
Response: Thanks for your comments. We have corrected it accordingly.

5. Lines 136-161: the results of the second part show that the basic mechanism of Tig-R and Tig-S exhibiting two different energy demand patterns is mainly caused by NUO-mediated NADH dehydrogenase. The above results do not have a strong logical relationship with the following results; therefore, I suggest that the authors rearrange this part of the results.

Response: Thanks for your comments. As you suggested, we have rearranged the results section and separated the energy demand-related results as a new **Fig. 2**.

6. Figure 2D-2E: the authors selected several strains of clinical Tig resistant bacteria, why not select several strains of clinical Tig sensitive bacteria as well for conducting the assays?

Response: Thanks for your comments. We have supplemented several strains of clinical Tig sensitive bacteria. As shown in **Fig. S7**, Met could not resensitize *tet*(X)-negative bacteria to Tig treatment.



Supplementary Fig. 7. Met failed to resensitize tet(X4)-negative bacteria to tigecycline.

*Percent survival of four tet(X4)-negative clinical strains, including E. coli G6, E. coli G92, K. pneumoniae KPC C14 and K. pneumoniae D120, in the presence of Tig, Met (20 mM) or both. Data were displayed as mean ± SEM, and statistical significance was determined by unpaired t-test. *P < 0.5, ns, not significant.*

7. Line 215: PMF has two components, ΔΨ and ΔpH, therefore why measure ΔΨ only and not ΔpH? The measure of ΔpH should be added. When testing ΔΨ, positive control should be added. In addition, the authors mentioned that exogenous methionine (Met) can enhance the uptake of tigecycline by upregulating the ΔpH component of the bacterial proton motive force (PMF). Could the authors discuss the reasons why Met facilitates the uptake of tigecycline but does not exhibit a synergistic effect with it?

Response: Thanks for your comments. We have measured ΔpH using BECEF-AM probe, and CCCP was added as a positive control in both ΔΨ and ΔpH measurement **(Figure 4C and S12C)**. In according with our previous results, bacterial ΔpH compensated mutually with ΔΨ dissipation under Met + Tig treatment. The action of Met is mainly dependently on the reprogramming of a series of resistance-related response initiated by bacterial populations under antibiotic exposure, rather than acting as a single enzyme inhibitor or active competitive site. The action could not be characterized through conventional fractional inhibitory concentration index (FICI) method, which was used to assess the synergistic bacteriostatic rather than bactericidal activity.

We reasoned that Met-derived Tig uptake was just an initial event, which incurred the subsequent series of resistance responses under a large amount of Tig. At this time, the availability of Met became a crucial factor in promoting methylation modification, thereby regulating the expression of *tet*(X4) gene through 5mC methylation. This conclusion can be supported by the evidenced results that only Met was added previously within 20 mins or added with Tig at the same time, the re-sensitization effect of combination treatment (Met and Tig) could be observed **(Fig. 3C and S8)**.

8. Why did *E. coli* J53 be selected instead of *E. coli* B3-1-(*tet*(X4)) or *E. coli* DH5α- (pUC19-*tet*(X4)), which had been phenotypically validated before, when the knockout bacteria were selected for testing?

Response: Thanks for your comments. *E. coli* B3-1 (*tet*(X4)) is a drug-resistant clinical isolate, which was difficult to carry out knockout-related experiments. In contrast, *E. coli* J53 is a widely used recipient strain that readily accepts a plasmid carrying *tet*(X4) from *E. coli* B3-1 (*tet*(X4)) through conjugation. Also, we proved that Met supplementation potentiated Tig activity against *E. coli* J53 (*tet*(X4)) **(Fig. S15A)**. Thus, *E. coli* J53 was selected for the following validation analysis.

9. When testing Minimum inhibitory concentration (MIC), quality control strains need to be added.

Response: Thanks for your comments. We have added the quality control strain *E. coli* 25922 in MIC testing, as shown in **Supplementary Table 1**.

10. Some bar graphs are presented in an inverted format, while others are in a standard format. Is there a specific reason for this variation? A uniform presentation of the bar graphs might improve readability for the readers.

Response: Thanks for your comments. We have tried to unify the presentation of most bar graphs in the revised manuscript. An inverted format was only used in the survival analysis to better reflect the reduction of bacterial loads under different treatments.

Reviewer #3 and #4

The work by Fang and collaborators highlights the importance of epigenetic regulation in bacterial metabolism and establishes the mechanisms underlying regulation through the methionine-mediated TetX regulon, which enhances the efficacy of the antibiotic tigecycline. Together, the findings presented in this article underscore bacterial biochemistry and metabolic homeostasis between tigecycline-sensitive and resistant strains, through a series of well-designed metabolomic and transcriptomic experiments. Additionally, the article addresses the regulation of tetX gene expression. The results presented in this article demonstrate a novel strategy in which methionine has great potential as an adjuvant targeting infections caused by multidrug-resistant bacteria containing the tet(X) resistance-associated gene.

This work is definitely significant, as Fang and collaborators provide evidence for reversing resistance in tetX+ strains through methionine supplementation, creating an opportunity for research in developing adjuvants from metabolites that enhance the effect of antibiotics. Even though components that enhance the bactericidal activity of tigecycline have been described, the mechanisms associated with this effect are not well understood. In this article by Fang and collaborators, the authors delineated the specific metabolic pathways involved that can be used to enhance the efficacy of an antibiotic, highlighting a close relationship between metabolic homeostasis and the final fate of the bacterial cell.

Response: Thanks for your insightful and valuable comments for improving our manuscript. We have revised our manuscript thoroughly based on your comments. All changes are highlighted by red in the revised manuscript.

• Does the work support the conclusions and claims, or is additional evidence needed?

Throughout the article, the work presents clear evidence for its claims and conclusions. The experimental design reflects the complexity and relevance of this study. However, to provide the reader with greater understanding, we suggest the following:

• Expand on the rationale for choosing a *Klebsiella pneumoniae* strain to introduce the tetX4 constructs.

Response: Thanks for your comments. In fact, *tet*(X4)-positive *Klebsiella pneumoniae* strain used in this study is a clinical isolate from pork samples. This would be more conducive to evaluating the clinical use potential of Met and the detailed information about this strain can be found in previous work from our lab[1].

1. Li, Y. et al. Emergence of *tet*(X4)-positive hypervirulent *Klebsiella pneumoniae* of food origin in China. *LWT* 173, 114280 (2023).

• In the methodological description of the transcriptome that references bibliography 42 of the article, the cut-off used for the fold change is 2. Why is a lower cut-off used for this work?

Response: Thanks for your comments. To better monitor the changes of metabolic pathways between drug-resistance and sensitive bacteria, we expanded the screening scope of differentially expressed metabolic genes through using a lower cut-off.

• Regarding Fig. 3, panels M and N, what explanation can be inferred if, in the presence of methionine and tigecycline, the transcripts of the tetX4 gene seem to disappear but can still be detected at the protein level under the same conditions (methionine and tigecycline present)?

Response: Thanks for your comments. We performed three independent replicates of these experiments and obtained similar results. In fact, the gene transcription and translation levels are sometimes not be completely synchronized, owing to various and complex reasons. Herein, we speculated that at 6 h of treatment, when the number of cells has shown a great

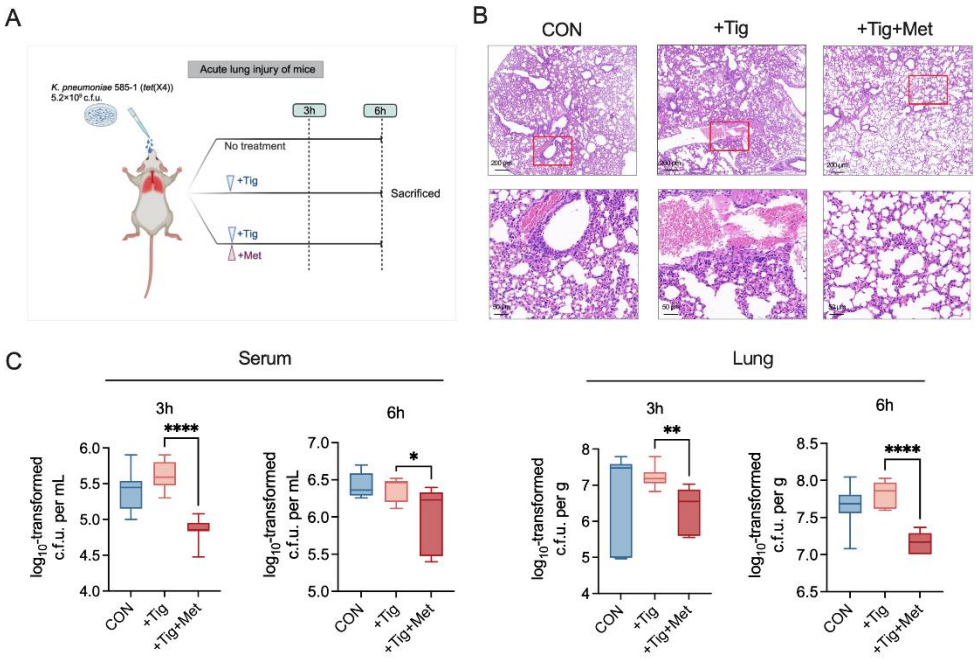
difference, the *tet*(X4) mRNA may have been degraded when the protein generation reached its peak, or Tet(X4) protein were well steady and had a strong affinity to antibody, therefore a small amount of protein has a strong detection signal.

• At what time points was relative expression determined in Fig. 3, panel G? Were the 6 hours maintained?

Response: Thanks for your comments. We extracted bacterial total RNA after 6 h treatment and normalized RNA concentration before the RT-PCR experiment. The detailed experimental details have been added in the revised manuscript.

• The authors describe an acute lung infection model in mice infected with K. pneumoniae 585-1 positive for tet(X4). However, a concern regarding this model lies in the intraperitoneal infection route with K. pneumoniae and its subsequent evaluation of lung damage caused 6 hours post-infection. This result does not represent a validated model for studying lung acute lung damage to better approach the infectious process caused by K. pneumoniae.

Response: Thanks for your comments. We have supplemented an acute lung infection model through a more appropriate infection route, rather than intraperitoneal inoculation. As shown in **Fig. S22**, to establish a representative model of acute lung damage, mice were given *K. pneumoniae* 585-1 (*tet*(X4)) via nasal drip infection route with a higher dose and the subsequent bacteria loads of lungs and serum were evaluated at 3 and 6 h post infection, as well as lung pathology sections at 6 h. It is noteworthy that Met plus Tig effectively reduced bacterial loads both in lungs and serum of mice, and alleviated inflammatory damage.



Supplementary Fig. 22. Met plus Tig is effective against K. pneumoniae in an acute mouse lung infection model.

(A) *The experimental protocols for assessing the potentiation of Met to Tig in an acute lung infection model. Tig, 20 mg/kg. Met, 100 mg/kg.* ***(B)*** *Hematoxylin and Eosin (H&E) staining of lungs from mice at 6 h post infection. (Scar bar, 200 μm & 50 μm).* ***(C)*** *Bacterial loads of infected mice lung and serum at 3 and 6 h. The mice were randomly divided into three groups (n = 10 per group), including K. pneumoniae 585-1 (tet(X4)), Tig, Tig + Met groups. Mice were given K. pneumoniae 585-1 (tet(X4)) via nasal drip infection route (5.2*10^9 CFU/mL, 50 μL per mice). Data were displayed as mean ± SEM, and statistical significance was determined by unpaired t-test. *P < 0.05, **P < 0.01, ****P < 0.0001.*

Minor revisions:

• The quality of the legends in Fig. 1 is poor, particularly panels D and E, which are not clearly visible.

Response: Thanks for your comments. We have replaced figures with higher quality in the revised manuscript.

• In Fig. 2, panel A, the legends are misaligned.

Response: Thanks for your comments. We have corrected it accordingly.

• In Fig. 3, panel N, the legend of the detected protein is missing.

Response: Thanks for your comments. We have added this missing information.

• The quality of the legends in Fig. 4 is poor, particularly panels A, B, C, J, and K.

Response: Thanks for your comments. We have replaced figures with higher quality in the revised manuscript.

• Line 34: Mention which infection models in animals were used. Lines 650-651: Correct "K pneumonias" to "K. pneumoniae". Line 651: Does the infection route refer to intraperitoneal? Please specify.

Response: Thanks for your comments. We have corrected these points accordingly.

Is the methodology sound? Does the work meet the expected standards in your field? Yes, the methodology is solid, and the experiments are complementary, allowing the reader to resolve most doubts. However, it is necessary to use a representative model of acute lung infection with K. pneumoniae to evaluate the adjuvant activity of methionine using the antibiotic tigecycline.

Response: Thanks for your comments. We have re-constructed an acute lung infection with *K. pneumoniae* model by nasal drip infection route. The results indicated that Met plus Tig is effective against *K. pneumoniae* 585-1 (*tet*(X4)) in a murine model of infection **(Fig. S22)**.

Is there enough detail provided in the methods for the work to be reproduced? Yes, the experiments are described in detail, and when the text does not explicitly state the details, a reference is provided for the specifications, as in the case of the construction of puc19.

Response: Thanks for your valuable comments for improving our manuscript.

Reviewer #5

The manuscript reports on a surprising discovery that resistance against the antibiotic tigecycline is mitigated through additional supply of methionine. The authors report several interesting data on the physiology of methionine treated and untreated cells and show that met serves as sensitizer of tigecycline susceptibility. This is in line with recent findings of methionine increasing general susceptibility against macrolide(!) antibiotics in Streptococcus suis (PMID: 38230928)

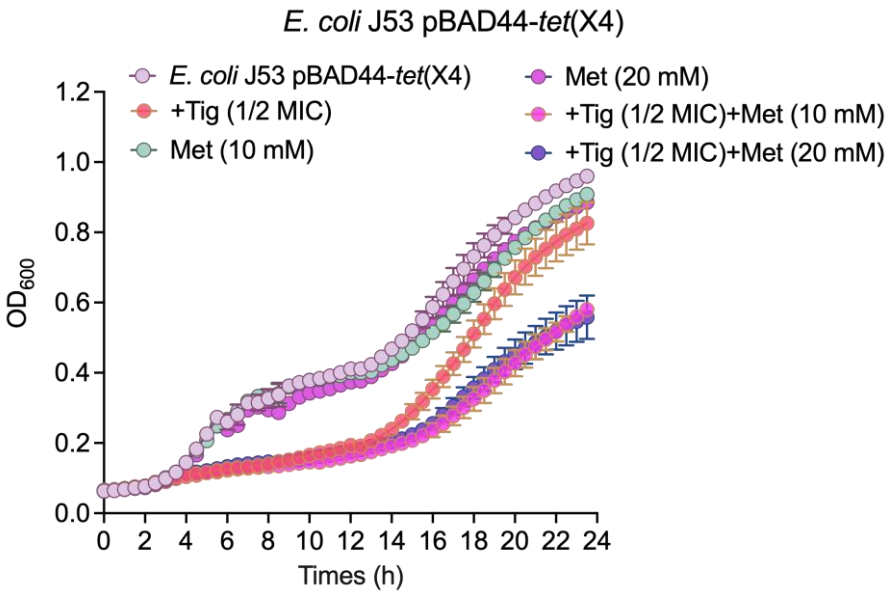
The observation is very interesting and the authors provide compelling data for the direct link between methionine and increased susceptibility. However, there are some concerns in respect to the actual mechanisms and whether it is specific (as proposed by the authors and in the title) or a more general, multi-layered mechanisms of methionine (in line with PMID: 38230928).

Response: Thanks for your insightful comments for improving our manuscript. We have revised our manuscript thoroughly based on your comments. All changes are highlighted by red in the revised manuscript.

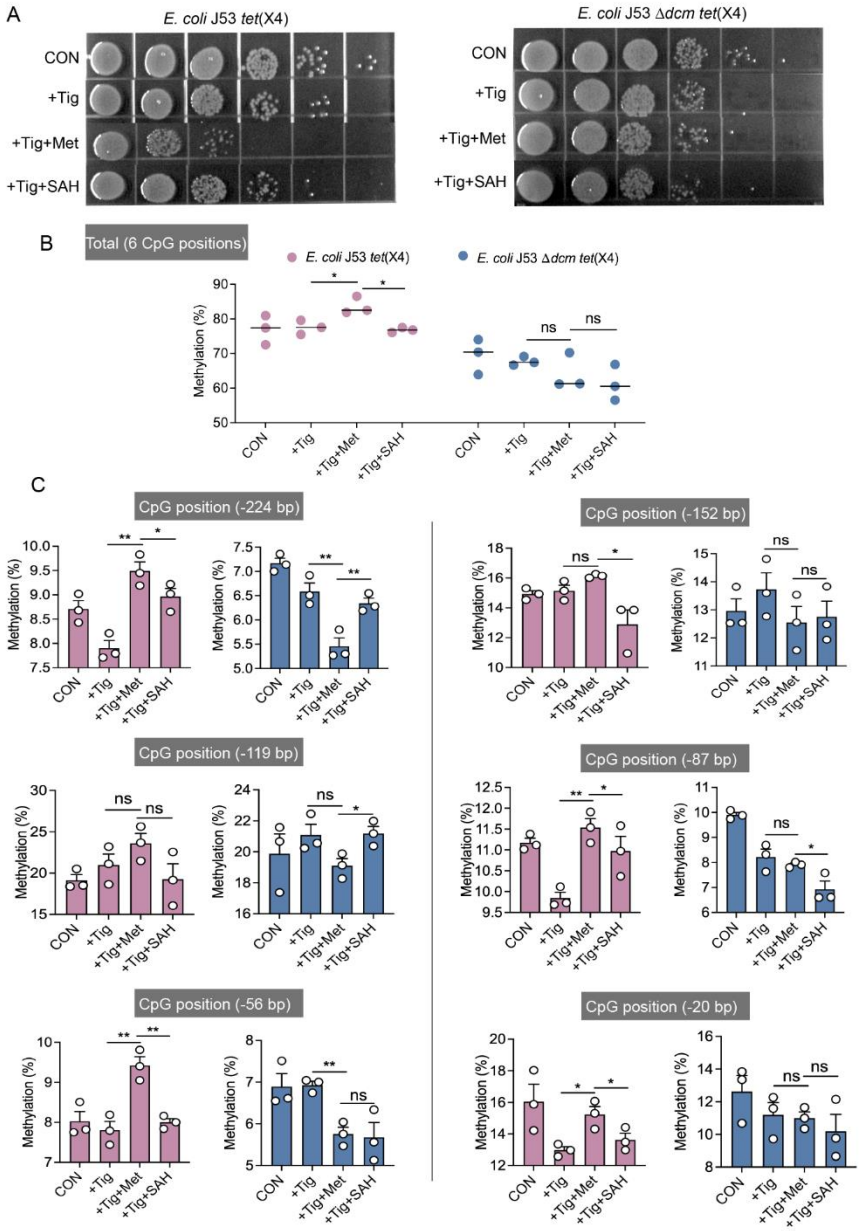
(1) methylation mechanism:

Since pUC19 use a high copy vector, I am particularly interested by the question, how the Met effect plays out in another, low copy vector. My feeling would be that this effect should be less pronounced, as methylation does not become a limiting factor anymore in a low copy plasmid. At a broader level, I am a generally puzzled that a vector of ~4-8 kb size (even if present at high copy, i.e, ~20 copies) would make a difference. This makes maybe ~100-200 kb extra DNA, which is less than 10% of the total genome (E coli. 3,400 kb!) that needs to be extry methylated. This less than 10% of total DNA and should not make a difference. How does the complete methylation pattern of E. coli actually look like under these conditions? Moreover, would the effect be completely eradicated in a vector that does not carry dcm motifs?

Response: Thanks for your valuable comments. We have constructed a low-copy vector with *tet*(X4) gene, which has been well-documented that conferring Tig-resistance in mobile plasmid, to investigate whether this effect was also pronounced. Notably, as shown in the Figure below, the growth curves of *E. coli* J53 pBAD44-*tet*(X4) was mostly restrained under the Tig + Met treatment, indicating that the action of Met is also applicable for low copy vector.

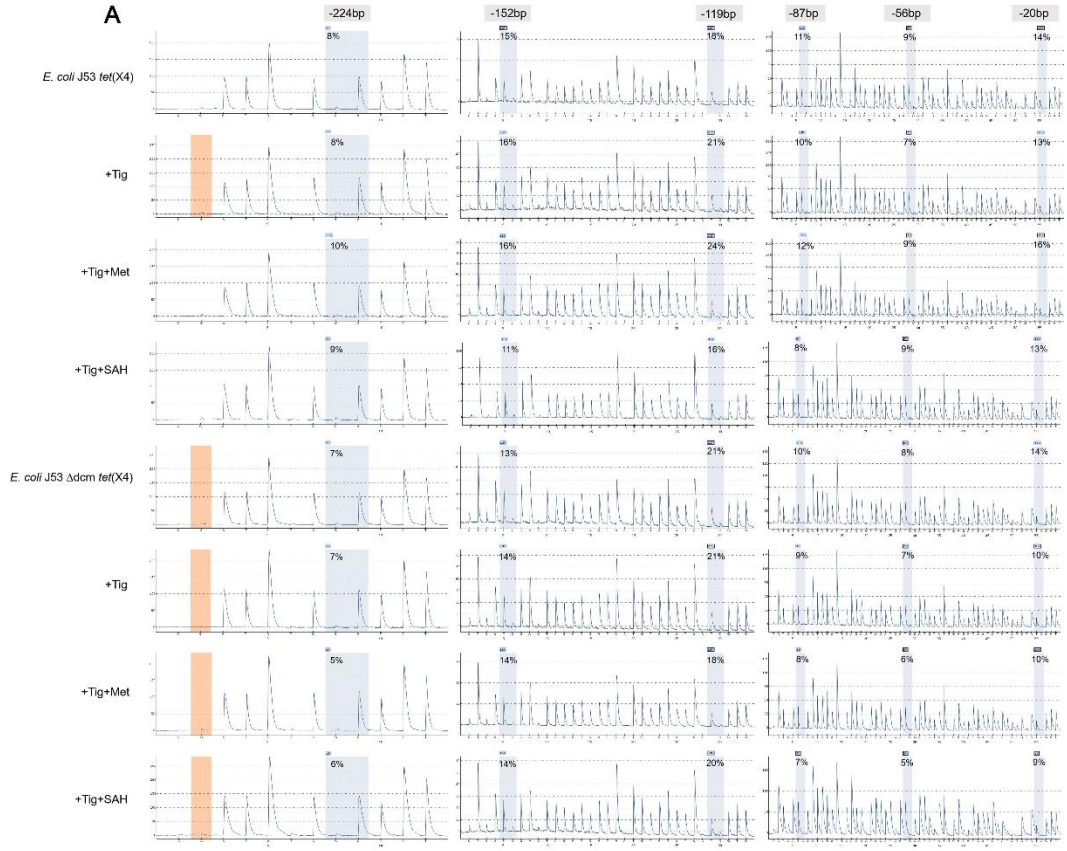


Additionally, *dcm* gene was located on the bacterial chromosome, not on the vector, which participated in 5mC methylation modification. In the revised manuscript, we constructed a *dcm*-deficiency *tet*(X4)-positive *E. coli* J53 Δ*dcm* in the bacterial genome. Our results demonstrated that the methylation pattern of *tet*(X4) promoter region were generally suppressed in the Δ*dcm* strain, and the effectiveness of Met + Tig treatment was also abolished accordingly **(Fig. S15 and S16,** as shown below**)**.



Supplementary Fig. 15. Deletion of dcm gene abolishes the potentiation of Met to Tig against tet(X4)-carrying bacteria.

(A) *Plate counting photograph of E. coli J53 tet(X4) and E. coli J53 Δdcm tet(X4) in the presence of Tig, Tig+Met, Tig+SAH. Tig,16 μg/mL. Met, 20 mM. SAH, 10 mM.* ***(B)*** *The total methylation rate of 6 CG sites in the promoter region of tet(X4) gene.* ***(C)*** *The methylation rate of specific CG site in the promoter region of tet(X4) gene in E. coli J53 tet(X4) (purple) and E. coli J53 Δdcm tet(X4) (blue). Experiments were performed with three biological replicates. Data were displayed as mean ± SEM, and statistical significance was determined by unpaired t-test. *P < 0.05, **P < 0.01, ns, not significant.*



B

Assay		
	Position	Sequence
Before Bisulfite Treatment	-224bp	GTAGCGTGATTGAACATACTTATATAATTGT
	-152bp/-119bp	TTCACGGAACAGATGACAAAATTTGGCACTATAAAAACGCTGG TGTATTAATTGAGAAAATACA
	-87bp/-56bp/-20bp	AATACGAAATTAATTACTCTTGAAGGAACAGGACACGAATTGC ACTTTGAAGATTGGACAACAATAATTGACGGAATTGAAAAACA TATAAATG AGC
Sequence to analyze	-224bp	ATAACRTAATTAAACATACTTATATAATTAT
	-152bp/-119bp	TTTAYGGAATAGATGATAAAATTTGGTATTATAAAAAYGTTGG TGTATTAATTGAGAAAATATA
	-87bp/-56bp/-20bp	AATAYGAAATTAATTATTTTTGAAGGAATAGGATAYGAATTGT ATTTTGAAGATTGGATAATAATAATTGAYGGAATTGAAAAATA TATAAATG AGT

Supplementary Fig. 16. Bisulfite sequencing information in the promoter regions of tet(X4) gene in E. coli.

*(**A**) Representative methylation rates in six CG sites under different treatments were listed. Three biological replicates were performed. **(B)** The detected CG sites of the promoter regions of tet(X4) in E. coli J53 and E. coli J53 Δdcm (marked with yellow).*

(2) alternative hypotheses:

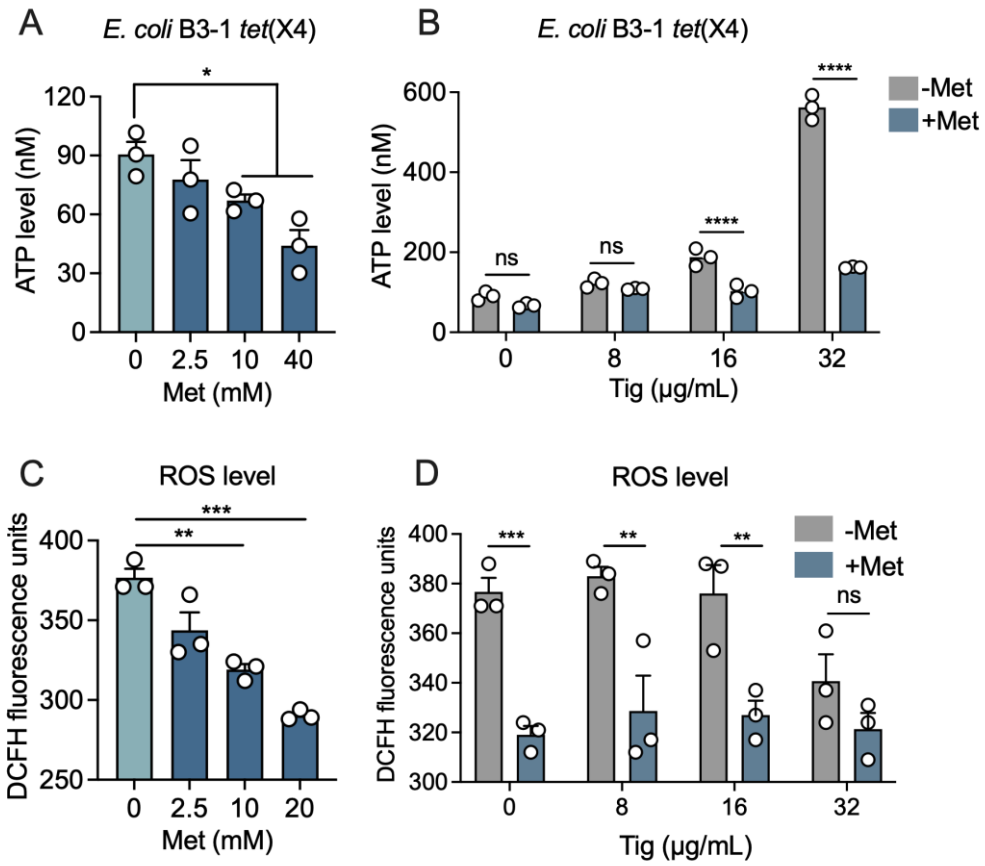
As much, as I like the idea of Met being important for dcm methylation, methionine could also work at other levels. What about the recent discovery that methionine works through decreasing efflux pump expression and general ROS stress sensitizer in Streptomyces reported by other authors recently (PMID: 38230928)? Could supply of methionine cause similar unspecific effects and increase ROS effects coming from unfruitful H2O2 production from tetX4, etc.? I don't think that the dcm expression/titrating experiment provides suffcient evidence that it is specific dcm patterns that cause the effect (it could be simply more SAM available of other biochemistry, etc.).

Response: Thanks for your comments. The over-production of ROS could result in bacterial death in general, especially under bactericidal antibiotics treatment. However, combined with our results about ROS & ATP production, we further confirmed that Met-mediated mechanism in Gram-negative *E. coli* was more likely to be unique rather than universal, which was absolutely differed from that in previously studied Gram-positive *Streptococcus suis* (PMID: 38230928). As shown in **Fig. S18**, both ATP and ROS generation were decreased under Tig + Met treatment in *E. coli* B3-1 *tet*(X4), which was opposed to the similar treatment by tylosin + Met in *Streptococcus suis*. Based on this, we inferred that Tig + Met mediated killing is attributed to the specific metabolic reprogramming and 5mC methylation modifications, rather than a general ROS-incurred cell death in a short time.

Additionally, to further investigate whether the mechanism is specific, we transferred *tet*(X4)-positive plasmid (from clinical-isolated *E. coli* B3-1 *tet*(X4)) to engineered strain *E. coli* J53 or *E. coli* J53 Δ*dcm*. Consistently, Met supplementation potentiates Tig activity against *E. coli* J53 *tet*(X4), this efficacy disappeared in *E. coli* J53 Δ*dcm tet*(X4) **(Fig. S15A)**. Consistently, the total cytosine methylation rates and 5mC methylation modification at 6 CG sites in the *tet*(X4) promoter region were reduced in *E. coli* J53 Δ*dcm tet*(X4) despite of the addition of Met **(Fig. S15B and S15C)**. These results collectively demonstrated that Met-

derived 5mC methylation modification in the promotor region of *tet*(X4) gene was critically responsible for the potentiation of Met to tigecycline against *tet*(X4)-positive *E. coli.*

To highlight this point, we have revised our manuscript as follows.



Supplementary Fig. 18. Met supplementation reduces ATP and ROS production in E. coli B3-1 (tet(X4)).

(A) *ATP levels of E. coli B3-1 (tet(X4)) in the presence of increasing concentrations of Met.* ***(B)*** *ATP levels of E. coli B3-1 (tet(X4)) in the presence of increasing concentrations of Tig with or without Met (20 mM).* ***(C)*** *ROS levels of E. coli B3-1 (tet(X4)) in the presence of increasing concentrations of Met.* ***(D)*** *ROS levels of E. coli B3-1 (tet(X4)) in the presence of increasing concentrations of Tig with or without Met (20 mM). Experiments were performed with biological replicates. Data were displayed as mean ± SEM, and statistical significance was determined by unpaired t-test. *P < 0.05, **P < 0.01 ***P < 0.001, ****P < 0.0001, ns, not significant.*

In summary, the data presented in the manuscript do not show that dcm 5mC methylation PRECISELY modulates *tet*(X) resistance expression. This is all interesting data, but not bolstered by any mechanistic explanation or sufficient controls/experiments by the authors. The authors are well-adviced to not over interpret their data (and overstate their findings, e.g. in the title/abstract). Especially, since the effect of Met in vivo is probably multi-layered - and eventually not specific (see PMID: 38230928), I believe that this needs to be reflected in the abstract and throughout the manuscript.

Response: Thanks for your valuable comments. Combined with bisulfite methylation sequencing, knockout related experiments and ATP/ROS analysis **(Fig. S13, 14, 15, 16 and S18)**, we concluded that the potentiation of Met to Tig against *tet*(X4)-positive bacteria in our study was specific rather than universal. Actually, we have quite different experimental settings compared with the study (PMID: 38230928), including strains (Gram-negative *E. coli* & Gram-positive *Streptococcus suis*), antibiotics (Tig & macrolides), co-incubation times, antibiotic resistance genes/phenotypes and so on. Certainly, we speculate that Met-mediated methylation plays an important role in this process, but it may not be the only one. Other possible mechanisms have been described in the Discussion section, such as immunomodulatory effect. These points have been highlighted in the revised manuscript.

To highlight this point, we have revised our manuscript as follows.

In E. coli B3-1 (tet(X4))-infected macrophages RAW264.7 cells, we found that the combination of Met and Tig significantly reduced the loads of intracellular bacteria, and this process was accompanied by an increased intracellular accumulation of Met ***(Fig. S19)***
(Line 309-312)

In fact, Met is also complicated in T cell activation and differentiation, such as manipulating epigenetic reprogramming in CD4($^{+}$) T helper (Th) cells. Furthermore, sufficient Met is critical for suppressing Th17 cell proliferation and the subsequent cytokines production, and the underlying mechanism also involves methylation modification (increased histone H3K4 methylation). Therefore, we speculated that alleviated inflammatory response in Tig plus

Met-treated mice can be explained by not limited to the efficacy of combination therapy but also Met-mediated immunotherapy. **(Line 404-410)**

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):
The revised manuscript has addressed my previous concerns and I would recommend it for publication.

Reviewer #2 (Remarks to the Author):
After the first round of review, the authors revised the manuscript according to the reviewers' suggestions and answered the questions I had raised in the previous round of review in detail. Following revisions to the manuscript and the addition of experimental data, the overall quality of the paper has been largely improved. However, there are still several issues that need further consideration.
1. In the abstract, the authors state: "Mechanistically, we demonstrate that exogenous Met promotes intracellular tigecycline accumulation by upregulating the bacterial proton motive force (PMF). Meanwhile, Met accelerates its conversion to S-adenosyl-L-methionine, a key methyl donor, thereby enhancing 5mC methylation in the promoter region of the tet(X4) gene and reducing its expression. This implies that changes in PMF are also one of the mechanisms by which tigecycline susceptibility is restored, and that these changes occur in parallel with methylation modifications. This appears inconsistent with the authors' response in this round. If the authors intend to demonstrate that increased PMF enhances tigecycline uptake, it would be essential to include data on intracellular tigecycline accumulation measured at different time points. In the absence of such data, I recommend a more precise explanation of the mechanism through which Met restores tigecycline susceptibility.
2. Given that the expression of resistance enzymes is invariably associated with an increased energy demand, it is reasonable to anticipate differences in energy consumption patterns between resistant and non-resistant bacteria under bacteriostatic or sub-bacteriostatic concentrations. However, it is crucial to emphasize that bacteria tend to perish at the MIC. Under these conditions, the metabolic differences observed between resistant and sensitive strains are inevitably inconsistent with those measured at sub-bacteriostatic concentrations, which are clearly of greater significance. Considering the constraints of time, cost, and workload, re-evaluating the data under sub-bacteriostatic concentrations may not be feasible. This, in my opinion, represents a limitation of this study.
3. " The action could not be characterized through conventional fractional inhibitory concentration index (FICI) method, which was used to assess the synergistic bacteriostatic rather than bactericidal activity." I find this explanation unconvincing.
4. Is there any specific reason for the inconsistency in the arrangement of the vertical axis in Figure 3A compared to other survival analysis plots?

Reviewer #3 (Remarks to the Author):
The authors have made clear observations, and the work shows convincing results that are suitable for publication. The representative model of lung infection provides a better approach for drawing conclusions from the research work.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5 (Remarks to the Author):
I appreciate the extra experiments to support the hypothesis that were provided by the authors and support publication of the study.

Reviewer #1 (Remarks to the Author)

The revised manuscript has addressed my previous concerns and I would recommend it for publication.

Response: Thanks for your positive comments.

Reviewer #2 (Remarks to the Author)

After the first round of review, the authors revised the manuscript according to the reviewers' suggestions and answered the questions I had raised in the previous round of review in detail. Following revisions to the manuscript and the addition of experimental data, the overall quality of the paper has been largely improved. However, there are still several issues that need further consideration.

Response: Thanks for your insightful comments for improving our manuscript. We have revised our manuscript thoroughly based on your comments. All changes are highlighted by red in the revised manuscript.

1.In the abstract, the authors state: "Mechanistically, we demonstrate that exogenous Met promotes intracellular tigecycline accumulation by upregulating the bacterial proton motive force (PMF). Meanwhile, Met accelerates its conversion to S-adenosyl-L-methionine, a key methyl donor, thereby enhancing 5mC methylation in the promoter region of the tet(X4) gene and reducing its expression. This implies that changes in PMF are also one of the mechanisms by which tigecycline susceptibility is restored, and that these changes occur in parallel with methylation modifications. This appears inconsistent with the authors' response in this round. If the authors intend to demonstrate that increased PMF enhances tigecycline uptake, it would be essential to include data on intracellular tigecycline accumulation measured at different time points. In the absence of such data, I recommend a more precise explanation of the mechanism through which Met restores tigecycline susceptibility.

Response: Thanks for your comments. We have measured intracellular Tig accumulation at

different time points in *E. coli* B3-1 (*tet*(X4)) under assumed treatments. Consistent with previous bacterial enumeration phenotype (**Supplementary Fig. 8c**), Tig accumulation elevated when Tig and Met added at the same time, but showed no change when adding Met later than Tig over 20 minutes. These results further confirmed our conclusions.

2.Given that the expression of resistance enzymes is invariably associated with an increased energy demand, it is reasonable to anticipate differences in energy consumption patterns between resistant and non-resistant bacteria under bacteriostatic or sub-bacteriostatic concentrations. However, it is crucial to emphasize that bacteria tend to perish at the MIC. Under these conditions, the metabolic differences observed between resistant and sensitive strains are inevitably inconsistent with those measured at sub-bacteriostatic concentrations, which are clearly of greater significance. Considering the constraints of time, cost, and workload, re-evaluating the data under sub-bacteriostatic concentrations may not be feasible. This, in my opinion, represents a limitation of this study.

Response: Thanks for your insightful comments. In this study, to comprehensively evaluate the effect of different antibiotic stresses on bacterial energy demands, we have tested a range of Tig concentrations that span from sub-bacteriostatic to bacteriostatic concentrations **(Figure 2)**, rather than focusing solely on sub-bacteriostatic concentrations. Regarding our findings of distinct energy demand patterns between resistant and non-resistant bacteria, more investigations are warranted to validate whether this pattern also apply to other antibiotics or under different conditions. These points have been highlighted in the **Discussion** section.

Our study revealed the differential metabolic characteristics and fluxes between tet(X)-negative and -positive E. coli, including distinct energy demand patterns under tigecycline exposure. More investigations are warranted to validate whether this patterns also apply to other antibiotics or under different conditions. **(Line 377-381)**

3." The action could not be characterized through conventional fractional inhibitory concentration index (FICI) method, which was used to assess the synergistic bacteriostatic

rather than bactericidal activity." I find this explanation unconvincing.

Response: Thanks for your comments. We have revised this description in the revised manuscript.

Furthermore, checkerboard assays indicated that Met and related metabolites did not exert direct drug-drug interaction with Tig (Supplementary Fig. 11a-g), implying that Met or Homoser did not function as conventional antibiotic potentiators which can be mechanistically explained as resistance enzyme inhibitors or active site competitors. **(Line 192-196)**

4.Is there any specific reason for the inconsistency in the arrangement of the vertical axis in Figure 3A compared to other survival analysis plots?

Response: Thanks for your comments. We have revised the vertical axis of Figure 3A accordingly.

Reviewer #3 (Remarks to the Author)
The authors have made clear observations, and the work shows convincing results that are suitable for publication. The representative model of lung infection provides a better approach for drawing conclusions from the research work.

Response: Thanks for your positive comments.

Reviewer #4 (Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thanks for your positive comments.

Reviewer #5 (Remarks to the Author):

I appreciate the extra experiments to support the hypothesis that were provided by the authors and support publication of the study.

Response: Thanks for your positive comments.