

Structural insights into context-specific inhibition of bacterial translation by macrolides

Corresponding Author: Dr Yury Polikanov

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)

The manuscript analyses the molecular details of macrolide mediated protein synthesis inhibition on the ribosome. It has been described earlier that the inhibition occurs on certain nascent peptide sequence motifs. It remained unexplained why specific amino acid residues lead to the inhibition. The current manuscript provides careful analysis of this issue. The experiments are well designed and performed. The conclusions are supported by solid data. The manuscript is very well written and figures well prepared. The study is important for antibiotic field, ribosome field and drug design in general. The minor comment I have is about figure 6. On panel I the orange color for residue -1 is confusing. As there is no stalling I would show it in blue.

Reviewer #2

(Remarks to the Author)

Syroegin et al. present eight high-resolution (2.65–2.8 Å) X-ray structures of *Thermus thermophilus* 70S ribosome–nascent-chain complexes (RNCs) — an undeniably heroic feat, given the technical difficulty of determining half a dozen independent ribosome crystals at this resolution. Using native-chemical ligation to create non-hydrolysable peptidyl- and aminoacyl-tRNA mimics, the authors determined structures of matched arrested and non-arrested complexes and uncovered a previously unseen “tilted” Lys-tRNA that shifts the α -amine away from the scissile bond, precluding catalysis. In erythromycin or telithromycin complexes, the penultimate basic residue of the nascent chain is forced into the A-site cleft, sterically blocking accommodation of the incoming Arg/Lys and explaining the Arg/Lys-X-Arg/Lys (“+X+”) stalling motif in line with ribosome-profiling data. These meticulously obtained structures close a long-standing mechanistic gap and provide a foundation for rational macrolide redesign.

The authors should also discuss potential species-specific effects and justify the resolution cutoff (why CC 1/2 is not used). With these modest additions, this heroic structural tour-de-force will meet the highest methodological standards and be of wide interest to ribosome biologists and antibiotic developers; I therefore recommend acceptance after minor revision.

Reviewer #3

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Macrolide context specificity was a breakthrough some years ago, but all details for this antibiotics action have not completely clarified up until today. The work of Polikanov et colleagues is basically a follow-up on the work of Daniel Wilson and Axel Innis (Beckert, B. et al. Structural and mechanistic basis for translation inhibition by macrolide and ketolide antibiotics. *Nat. Commun.* 12, 4466, 2021) that was published a few years ago. Here, there is an advanced study with many experimental advantages easily concluding what is getting wrong in the PTC when there is +X+ amino acid motif and a macrolide juxtaposed in the tunnel. Interestingly, the placement of +X+ in the Peptidyl Transferase Center, even without a macrolide, is already almost a step away from disaster, and macrolides only exacerbate that problem. The strongest indication in this case is that the penultimate arginine in the developing strand naturally prefers to accommodate not into the tunnel as we would anticipate, but surprisingly in the ribosome A site. In comparison to previous works, rather than using molecular dynamics simulations which are less relatively uninformative, here the authors show directly and experimentally what is getting wrong in the PTC. Again, interestingly, their structures show that the ribosome stalls, not because there are weird positions of the PTC nucleotides but rather because particular tRNAs cannot accommodate into the PTC.

The main advance that they made here is that they have reported control structures of the non-stalled complexes, allowing them to make direct comparisons with the stalled complexes structures and understanding precisely the structural differences.

The work is of high quality, with new and interesting data, and my suggestion is to be accepted for publication. Here are some minor points to be addressed before publication.

- Concerning the macrolides displacement in the presence of bulky residues, is it clear that not any conformational changes take place?
- In the case of a simple competition increasing one competitor concentration displaces the other? Is it true here?
- Usually, competition takes place for the same or a mutually exclusive site. Is it the case here or looks more like a noncompetitive inhibition?
- Line 386 in the text: are nearly indistinguishable... What does it mean to say that you perceive no difference or that the changes you do observe are not significant?
- Figure S2 could help readers to clarify easily the differences among macrolide used motifs and I think that it must be moved from supplementary to the main article.

Reviewer #4

(Remarks to the Author)

This study aims to elucidate the specific roles of macrolides, the ribosome, and tRNA in translating the +X+ motif. The authors assembled *Thermus thermophilus* 70S ribosomes with various peptidyl-tRNAs positioned in the P-site and A-site, and determined their X-ray structures. In the presence of the macrolide erythromycin (ERY) or ketolide telithromycin (TEL), the penultimate arginine residue of the P-site peptidyl-tRNA adopts an unusual conformation that partially obstructs the A-site cleft. This steric hindrance interferes with the accommodation of Lys-tRNA in the A-site. Such a conformation appears to be specific to lysine and arginine residues, likely due to their long and flexible side chains. In contrast, residues with shorter or more rigid side chains cannot adopt this obstructive conformation, providing a structural explanation for the ribosomal arrest at +X+ motifs in the presence of macrolides.

However, the major conclusions of this study have already been proposed by Beckert et al. (Nature Communications, 2021). The current work does not offer significant mechanistic advances beyond that prior study. While the authors do confirm the unusual conformation of the penultimate arginine via X-ray structures, their findings primarily serve to reinforce previously proposed mechanisms, rather than reveal new insights. The inclusion of various A-site aa-tRNAs in the structures is a useful extension, but does not fundamentally alter the understanding of +X+ motif-mediated arrest.

Major Concern:

Comparing the electron density across different figures, in Figure 1, the α -amino group of the A-site lysine appears to be well-supported by the density. However, in Figures 2 and 4, the α -amino groups poised for nucleophilic attack lie outside of the density, raising concerns about the accuracy of their modeled conformations—particularly in Figure 1. Given the local flexibility of this region, it remains difficult to definitively assess the correctness of these conformations.

Furthermore, the authors suggest that the flexibility of the +1 Lys side chain is insufficient to push the -1 Arg back into a normal conformation. But one may also ask: is the side chain flexibility of Lys insufficient to position the α -amino group in the correct geometry for nucleophilic attack? To better understand the roles of side chain flexibility, charge, and hydrophobicity, molecular dynamics simulations would be necessary. Simulations could reveal how amino acid properties influence stalling efficiency, calculate the likelihood of nucleophilic attack, and help determine whether the observed stalling behavior matches these probabilities. As such, the current work—like Beckert et al.—primarily reports an observed phenomenon but stops short of mechanistically confirming the cause.

Additional Points:

1. The manuscript includes too many keywords. Please consider reducing them for clarity and focus.
2. The Results section includes excessive background, especially the first part. The rationale for choosing the short ErmDL construct can be briefly summarized rather than extensively discussed.
3. Regarding Figure S2:
 - (1) In lanes 2 and 4, stalling is evident at the first R residue, whereas lane 6 shows no such stalling. What accounts for this difference?
 - (2) In lane 2, there is also strong stalling at the second R residue in the MRLR sequence. The band intensity is comparable to the first R. How is this interpreted?
 - (3) Lanes 2 and 4 (MRL) show stronger stalling than lane 6 (MRC). What does this suggest about the difference between leucine and cysteine at this position?
 - (4) In the MR vs. MRL comparison, MR shows a more intense band at the first nucleotide, while MRL shows a stronger second-band signal. Does this pattern align with the proposed arrest mechanism? Notably, arrest induced by catch-codons also shows stronger first-band signals. Could the authors comment on these observations to further validate their model?
4. In some structural figures, color differences between components are too subtle, making interpretation difficult. Please consider enhancing contrast to improve visual clarity.

Reviewer comments:

Reviewer #4

(Remarks to the Author)

The authors have addressed most of my concerns. However, I still have several suggestions:

1. Figure 1c vs. Figure 2d: It seems like that the conformation of the A76 residue of the P-site tRNA in Figure 1c differs from that shown in Figure 2d (the deep blue model). Please carefully verify whether this discrepancy is real or the result of visualization/rendering differences. I suggest not use deep blue and show the N and O atoms in Figure 1c.
2. Analysis of +X+ stalling in Figure S7: The intent of Figure S7 is to demonstrate that the stalling pattern for the +X+ motif is similar between ERY and TEL treatment. Instead of presenting four representative regions, I suggest performing a quantitative comparison, such as calculating the correlation coefficient between the two datasets. Furthermore, approximately half of the +X+ sites appear to exhibit no detectable stalling, which raises the question of whether additional sequence or structural context is required to induce stalling. Given the apparent overall correlation between ERY- and TEL-induced stalling at +X+ sites, it would be valuable to further analyze or discuss whether the presence of a +X+ motif alone is sufficient for stalling, or whether other factors modulate this effect.
3. The authors stated that the reported resolutions are based on an $I/\sigma(I)$ threshold of 1.0. However, Table S1 indicates that the $I/\sigma(I)$ values for some outer resolution shells range between 0.82 and 0.88, which appears inconsistent with the stated threshold.

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Response to Reviewer #1

Remarks to the Authors:

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The minor comment I have is about figure 6. On panel I the orange color for residue -1 is confusing. As there is no stalling I would show it in blue.

Response: We thank the reviewer for supportive and thoughtful assessment of our work. Indeed, in panels A and I of Figure 6, we highlighted the penultimate (-1) residue in orange to emphasize that this position is inherently problematic and contribute to ribosome stalling when combined with other arrest-promoting determinants, such as an incompatible A-site amino acid and a bound macrolide. Although the specific sequence in panel I does not result in stalling, we deliberately maintained the orange color to underscore the latent arrest potential at this position under different contextual conditions. To avoid confusion, we have added clarifying text in the figure legend to explain this choice and explicitly state that the coloring reflects mechanistic roles and not necessarily outcomes in each depicted scenario.

Response to Reviewer #2

Remarks to the Authors:

Syroegin et al. present eight high-resolution (2.65–2.8 Å) X-ray structures of Thermus thermophilus 70S ribosome–nascent-chain complexes (RNCs) — an undeniably heroic feat, given the technical difficulty of determining half a dozen independent ribosome crystals at this resolution. Using native-chemical ligation to create non-hydrolysable peptidyl- and aminoacyl-tRNA mimics, the authors determined structures of matched arrested and non-arrested complexes and uncovered a previously unseen “tilted” Lys-tRNA that shifts the α -amine away from the scissile bond, precluding catalysis. In erythromycin or telithromycin complexes, the penultimate basic residue of the nascent chain is forced into the A-site cleft, sterically blocking accommodation of the incoming Arg/Lys and explaining the Arg/Lys-X-Arg/Lys (“+X+”) stalling motif in line with ribosome-profiling data. These meticulously obtained structures close a long-standing mechanistic gap and provide a foundation for rational macrolide redesign.

The authors should also discuss potential species-specific effects and justify the resolution cutoff (why CC 1/2 is not used). With these modest additions, this heroic structural tour-de-force will meet the highest methodological standards and be of wide interest to ribosome biologists and antibiotic developers; I therefore recommend acceptance after minor revision.

Response: We are grateful for the reviewer’s enthusiasm about our work. Regarding the species-specificity of the stalling mechanism, we have already addressed this point at the end of the Discussion section by noting that the +X+ motif-induced arrest is observed in multiple bacterial species and even in sensitized eukaryotic ribosomes (see ref. 22), indicating that the mechanism is broadly conserved.

As for the resolution cutoff, rather than using $CC1/2 = 0.5$ (a threshold commonly used in the literature to define the outer resolution shell) or even $CC1/2 = 0.143$ (cutoff used in cryo-EM), we chose to report resolution using the more traditionally accepted criterion of $I/\sigma I = 1.0$. This approach has been widely used in X-ray crystallography to determine the resolution limit and is generally consistent with a $CC1/2$ of approximately 0.5 across all datasets. Although we did calculate $CC1/2$ values during data processing and used them to monitor data quality, our final reported resolutions are based on the $I/\sigma I = 1.0$ threshold. We have now clarified this explicitly in the revised Methods section.

Response to Reviewer #3

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Macrolide context specificity was a breakthrough some years ago, but all details for this antibiotics action have not completely clarified up until today. The work of Polikanov et colleagues is basically a follow-up on the work of Daniel Wilson and Axel Innis (Beckert, B. et al. Structural and mechanistic basis for translation inhibition by macrolide and ketolide antibiotics. Nat. Commun. 12, 4466, 2021) that was published a few years ago. Here, there is an advanced study with many experimental advantages easily concluding what is getting wrong in the PTC when there is +X+ amino acid motif and a macrolide juxtaposed in the tunnel. Interestingly, the placement of +X+ in the Peptidyl Transferase Center, even without a macrolide, is already almost a step away from disaster, and macrolides only exacerbate that problem. The strongest indication in this case is that the penultimate arginine in the developing strand naturally prefers to accommodate not into the tunnel as we would anticipate, but surprisingly in the ribosome A site. In comparison to previous works, rather than using molecular dynamics simulations which are less relatively uninformative, here the authors show directly and experimentally what is getting wrong in the PTC. Again, interestingly, their structures show that the ribosome stalls, not because there are weird positions of the PTC nucleotides but rather because particular tRNAs cannot accommodate into the PTC.

The main advance that they made here is that they have reported control structures of the non-stalled complexes, allowing them to make direct comparisons with the stalled complexes structures and understanding precisely the structural differences. The work is of high quality, with new and interesting data, and my suggestion is to be accepted for publication.

Here are some minor points to be addressed before publication:

1. *Concerning the macrolides displacement in the presence of bulky residues, is it clear that not any conformational changes take place?*

Response: Excellent point! As shown in Figure 5, when bulky amino acids such as Phe are accommodated in the A site, the electron density for the NPET-bound macrolide vanishes, indicating that the drug has been displaced. Structural alignment of the ribosome before and after this displacement reveals no significant conformational changes in the PTC or surrounding rRNA. This suggests that macrolide eviction is a direct consequence of steric conflict with the incoming residue, mediated by the penultimate Arg/Lys residue of the nascent peptide, rather than the result of broader structural rearrangements of the key nucleotides in the PTC and NPET. We have revised the main text to emphasize this point.

2. *In the case of a simple competition increasing one competitor concentration displaces the other? Is it true here?*

Response: Theoretically, yes – but with important nuances. In the classical direct competition, two molecules bind to the same or overlapping binding sites, and increasing the concentration of one typically results in the gradual displacement of the other. In our system, however, the incoming aa-tRNA and the macrolide do not compete for the same physical site on the ribosome. The macrolide binds within the nascent peptide exit tunnel, while the aminoacyl moiety of the tRNA binds in the A-site cleft of the peptidyl transferase center. Critically, this competition becomes possible only in the presence of a penultimate Arg or Lys residue in the nascent chain. These long and

flexible side chains are capable of bridging the distance between the A site and NPET, creating an indirect steric connection between the incoming aa-tRNA and the macrolide. In this case, the -1 residue acts as a mediator that enables this competition to occur. The outcome of such competition depends not only on the relative affinities of the macrolide and the amino acid moiety of the aa-tRNA for their respective binding pockets but also on the relative affinity of the -1 Arg/Lys residue for either its binding pose within the NPET or an alternative conformation in the A-site cleft.

Therefore, this is not a classical case of concentration-driven displacement. Increasing tRNA concentration alone would not necessarily dislodge the macrolide or relieve arrest. The arrest phenotype is context-specific and emerges from the three-way interplay between the nascent peptide, the incoming tRNA, and the ribosome-bound macrolide. These multiple interdependent components complicate the predictions even further, as the final outcome depends on a delicate balance between molecular affinities, nascent peptide sequence, and ribosomal geometry.

3. *Usually, competition takes place for the same or a mutually exclusive site. Is it the case here or looks more like a noncompetitive inhibition?*

Response: No, this is not the case here: the macrolide and the incoming aa-tRNA do not bind the same or even overlapping sites (see previous comment). However, the presence of one indirectly prevents the productive accommodation of the other through steric interactions mediated by the penultimate Arg/Lys residue of the nascent peptide. The system behaves as a form of context-specific, indirect competition that is enabled only when specific features of the nascent chain, namely a long, flexible basic side chain, are present. This behavior does not align with classical noncompetitive inhibition either, since the effect is not due to allosteric changes in the ribosome's active site but instead depends on spatial interference that only arises under specific conditions. We believe this represents a unique, sequence-dependent form of sterically mediated interference rather than a textbook example of competitive or noncompetitive inhibition.

If we were to classify the type of inhibition that macrolides exert in this system, it most closely resembles uncompetitive inhibition – a rare but well-defined model in enzymology, in which the inhibitor binds adjacent to the active site of an enzyme and require a substrate to be present prior to its binding, thereby forming a non-productive ternary complex (e.g., inhibition of inosine-monophosphate dehydrogenase by mycophenolic acid¹). Analogously, macrolide-induced ribosome stalling occurs only after a specific nascent peptide sequence (+X+ motif) has been synthesized inside the ribosome. In the absence of this context, macrolides do not block translation. Therefore, the macrolide acts on the functional enzyme-substrate complex (the ribosome engaged in translating a +X+ motif), consistent with the mechanistic logic of uncompetitive inhibition. We have revised the main text to include additional paragraph in the discussion section reflecting on important point.

4. *Line 386 in the text: are nearly indistinguishable... What does it mean to say that you perceive no difference or that the changes you do observe are not significant?*

Response: We appreciate the request for clarification. In this case, “*nearly indistinguishable*” refers to the absence of any significant or functionally meaningful differences in the conformations between the compared structures. We have revised the corresponding sentence in the main text to clearly reflect this interpretation.

5. *Figure S2 could help readers to clarify easily the differences among macrolide used motifs and I think that it must be moved from supplementary to the main article.*

Response: We appreciate the reviewer's suggestion and understand the motivation behind highlighting the sequence-dependent effects of macrolides. However, we feel that keeping Figure S2 in the Supplementary section is more appropriate. The content of this figure is inherently technical and supplementary in nature. It presents toeprinting results that confirm the system behaves as expected and that the assay conditions reproduce well-established stalling behavior. Specifically, the figure demonstrates that macrolide-dependent arrest persists even in a modified MRCK variant, where the classical ErmDL-derived MRLR motif has been altered at both the +1 and ultimate positions (Arg → Lys and Leu → Cys), but the +X+ stalling context is still maintained. This result validates that the sequence context remains arrest-competent and supports the interpretation of our structural findings. However, it is a secondary control that reinforces, rather than drives, the main mechanistic conclusions of the study. Similar experimental designs and findings have also been reported in earlier studies, including those by the Mankin group, further underscoring the confirmatory nature of this figure. For this reason, we prefer to retain Figure S2 in the Supplementary section.

Response to Reviewer #4

Remarks to the Authors:

This study aims to elucidate the specific roles of macrolides, the ribosome, and tRNA in translating the +X+ motif. The authors assembled Thermus thermophilus 70S ribosomes with various peptidyl-tRNAs positioned in the P-site and A-site, and determined their X-ray structures. In the presence of the macrolide erythromycin (ERY) or ketolide telithromycin (TEL), the penultimate arginine residue of the P-site peptidyl-tRNA adopts an unusual conformation that partially obstructs the A-site cleft. This steric hindrance interferes with the accommodation of Lys-tRNA in the A-site. Such a conformation appears to be specific to lysine and arginine residues, likely due to their long and flexible side chains. In contrast, residues with shorter or more rigid side chains cannot adopt this obstructive conformation, providing a structural explanation for the ribosomal arrest at +X+ motifs in the presence of macrolides.

*However, the major conclusions of this study have already been proposed by Beckert *et al.* (Nature Communications, 2021). The current work does not offer significant mechanistic advances beyond that prior study. While the authors do confirm the unusual conformation of the penultimate arginine via X-ray structures, their findings primarily serve to reinforce previously proposed mechanisms, rather than reveal new insights. The inclusion of various A-site aa-tRNAs in the structures is a useful extension, but does not fundamentally alter the understanding of +X+ motif-mediated arrest.*

Response: We respectfully disagree with the assessment that our study does not offer mechanistic advances beyond the previous work of Beckert *et al.*. While the earlier work provided foundational insights, it lacked structures of non-arrested control complexes (which we report here) and did not include A-site tRNA, making it impossible to determine the mechanistic origin of the ribosome stalling. Although Beckert *et al.* first observed the unusual conformation of the penultimate arginine in ErmDL, their work did not explain the origin of this conformation. Specifically, it remained unclear whether this rearrangement was due to the intrinsic properties of the ErmDL peptide or was induced directly or indirectly (allosterically) by the presence of a tunnel-bound macrolide. In that work, the authors used molecular dynamics (MD) simulations to mimic the accommodation of various aminoacyl-tRNAs into the A site, the tRNAs that were not directly visualized in any of their structures. The simulations predicted similar accommodation for Arg/Lys and also bulky aromatic residues such as Phe, and suggested that all of them would encounter similar steric issues entering the A site. However, this contradicts experimental data: ribosome profiling and toeprinting studies clearly show that ribosomes stall at +X+ motifs but not at +XY sequences (where Y is a bulky aromatic residue). Moreover, the reported MD simulations were unable to predict one of our key findings, that Phe accommodation leads to macrolide displacement. These inconsistencies further highlight the limitations of relying on computational simulations in the absence of structural or biochemical validation.

In contrast, our study experimentally addresses these questions by presenting high-resolution, matched structures of arrested and non-arrested ribosome-nascent chain complexes, all of which include both P- and A-site tRNAs. These structures reveal that the incoming aa-tRNA adopts a distinct, misaligned conformation in the presence of a macrolide and a penultimate Arg/Lys, resulting in catalytically unproductive geometry in the PTC. This macrolide-induced mispositioning of the A-site tRNA, unobservable in the prior work, appears to be the direct cause of translation arrest. Importantly, by

determining structures of the control non-arrested complexes, we dissect the individual roles of the tunnel-bound drug, the -1 residue in the peptide, and the +1 incoming amino acid, thus filling a crucial mechanistic gap left unresolved in prior studies.

Moreover, we determined the structure of a ribosome complex with Phe-tRNA accommodated in the A site revealing that the Phe side chain snugly fits into the A-site cleft and displaces the -1 Arg residue of the nascent chain into a position incompatible with macrolide binding, leading to drug eviction. This experiment provided direct structural evidence that competition between the incoming amino acid and the NPET-bound macrolide does occur, but critically, this is an indirect, allosteric form of competition mediated by the flexible Arg/Lys side chain in the -1 position of the nascent peptide.

Taken together, these new findings provide crucial mechanistic insight into why stalling occurs specifically at +X+ motifs (but not at +XY sequences) and conceptually expand our understanding of ribosome stalling. Our work reveals that the interplay between the nascent peptide and macrolide can modulate A-site selectivity – an unexpected and elegant example of allosteric communication within the ribosome.

Major Concern: Comparing the electron density across different figures, in Figure 1, the α -amino group of the A-site lysine appears to be well-supported by the density. However, in Figures 2 and 4, the α -amino groups poised for nucleophilic attack lie outside of the density, raising concerns about the accuracy of their modeled conformations, particularly in Figure 1. Given the local flexibility of this region, it remains difficult to definitively assess the correctness of these conformations.

Response: We thank the reviewer for careful examination of our structural data. The apparent weaker density in some maps may reflect some local flexibility but not modeling inaccuracies. Although we agree that the α -amino group in Figures 2 and 4 appears to lie somewhat outside the displayed density, this impression partially arises from the chosen viewing angle used to generate the figures. This particular orientation was selected to best illustrate the position of the lysine residue relative to the peptidyl-tRNA in the P site and to highlight the geometry and distance of the nucleophilic attack trajectory. Moreover, at a lower contour level, electron density is present around the α -amino group. However, we chose not to display the maps at levels below the standard 1σ threshold used for representation of X-ray maps, both to adhere to convention and to avoid visual clutter from surrounding density.

It is also important to emphasize that the α -amino group of the incoming amino acid does not exist in isolation within the ribosome: it is a covalently connected part of the aminoacyl-tRNA, and its position can also be determined from the established geometry of the lysine residue. In all our structures, the side chain of the lysine residue is clearly resolved and unambiguously positioned in the maps. As a result, the placement of the α -amino group is reliable, even in regions where the density may be locally weaker. Overall, we are confident that the modeled position of the α -amino group reflects the catalytically competent state observed in our control non-arrested complexes and supports the mechanistic conclusions of this study.

Furthermore, the authors suggest that the flexibility of the +1 Lys side chain is insufficient to push the -1 Arg back into a normal conformation. But one may also ask: is the side chain flexibility of Lys insufficient to position the α -amino group in the correct geometry for nucleophilic attack? To better understand the roles of side chain flexibility, charge, and hydrophobicity, molecular dynamics simulations would be necessary. Simulations could reveal how amino acid properties influence stalling efficiency, calculate the

likelihood of nucleophilic attack, and help determine whether the observed stalling behavior matches these probabilities. As such, the current work, like Beckert *et al.*, primarily reports an observed phenomenon but stops short of mechanistically confirming the cause.

Response: This is a thoughtful and important point. Indeed, we propose that the flexible aliphatic side chains of incoming Arg and Lys residues are insufficient to displace the penultimate Arg/Lys residue from its macrolide-induced position in the A-site cleft. This likely results from the relatively low affinity of these basic residues for the A-site cleft, which in turn fails to generate enough steric force to dislodge the penultimate residue in the presence of a tightly bound macrolide, which needs to be evicted in order for the -1 Arg/Lys to change its position. In contrast, when a bulkier and certainly more rigid aromatic residue such as Phe is accommodated into the A site cleft, it appears to exert sufficient force on the penultimate Arg/Lys residue to push it into a conformation incompatible with macrolide binding, leading to drug eviction. This observation suggests that the relative affinity and steric influence of the incoming residue, not just its presence, are key determinants of whether stalling occurs in the presence of a macrolide or not.

To address the reviewer's question directly: although the lysine side chain is flexible, successful nucleophilic attack requires that its α -amino group be precisely positioned relative to the carbonyl carbon of the peptidyl-tRNA in the P site. Additionally, the α -amino group must form a hydrogen bond with the 2'-OH of the A76 of the P-site tRNA to establish the proton relay ("proton wire") required for catalysis. This precise geometry is impossible to achieve structurally if the lysine side chain is not properly accommodated into the A-site cleft, especially when its positioning is sterically hindered by the penultimate residue under macrolide-bound conditions. Therefore, the answer is likely *"No, the side chain flexibility of incoming Lys is insufficient to position the α -amino group in the correct geometry for nucleophilic attack to occur"*.

We agree that, in this work, we do not experimentally measure the relative affinities of different amino acid side chains for the A-site cleft. Rather, we assumed that rigid bulky residues possess higher affinity and steric impact than the more flexible Arg/Lys side chains. While speculative, we believe this is a reasonable assumption based on structural observations and previous biochemical studies.

We also acknowledge the reviewer's suggestion to use molecular dynamics (MD) simulations. While MD can offer insights in many cases, we are cautious in applying it here. This is because the affinity of incoming side chains for the A-site cleft is likely governed largely by van der Waals, stacking, and hydrophobic interactions – forces that are often unaccounted for in conventional MD force fields, which predominantly emphasize electrostatics (e.g., H-bonds). As a result, MD simulations may not only fail to represent the nuanced balance of interactions that governs side chain accommodation in the A-site cleft formed by flat aromatic residues A2451 and C2452, but may also generate predictions that are misleading. For these reasons, we believe that direct biophysical measurements of binding affinities would provide a more reliable means to address this question, although such work falls outside the scope of the current structural study and the expertise in our labs.

Finally, we wholeheartedly agree with the reviewer that a more systematic investigation into how side chain flexibility, charge, and hydrophobicity influence stalling efficiency would be highly valuable. We view this as a compelling direction for future research, but one that is beyond the immediate scope of the current work, which focuses on defining the structural basis of +X+-dependent arrest.

Additional Points:

1. *The manuscript includes too many keywords. Please consider reducing them for clarity and focus.*

Response: We have reduced and streamlined the list of keywords to improve clarity and focus.

2. *The Results section includes excessive background, especially the first part. The rationale for choosing the short ErmDL construct can be briefly summarized rather than extensively discussed.*

Response: We appreciate the reviewer's feedback regarding the introductory portion of the Results section. While we agree that background information (especially in the Results section) should be concise, we felt it was critically important to clearly explain our rationale for choosing the short version of the ErmDL stalling peptide sequence. This explanation is essential not only for clarifying our experimental design but also for distinguishing our work from previous structural studies. Specifically, the full-length heptapeptide ErmDL (MTHSMRL-R) contains two distinct stalling determinants: (1) an N-terminal segment that interacts with the cladinose moiety of the macrolide in the exit tunnel, and (2) a C-terminal motif (MRL-R) that interacts with the PTC and is sufficient to cause stalling on its own. Beckert *et al.* demonstrated that each of these determinants can independently induce macrolide-dependent arrest. In this work, our goal was to focus exclusively on the C-terminal +X+ motif and dissect its structural contributions to stalling. To do so, we used a shorter version of the ErmDL peptide that isolates this stalling element and avoids potential confounding effects from the upstream N-terminal segment.

We felt that articulating this decision clearly in the Results section would be helpful, especially for readers less familiar with the intricacies of ErmDL-mediated stalling. However, we fully agree with the reviewer that some parts, particularly the extended discussion of shortcomings in previous cryo-EM studies, indeed could have been shortened or removed. In response, we have revised the text to streamline the corresponding part of the Results section and to focus more succinctly on the key rationale behind our model choice.

Regarding Figure S2:

3. *In lanes 2 and 4, stalling is evident at the first R residue, whereas lane 6 shows no such stalling. What accounts for this difference?*

Response: Indeed, in lanes 2 and 4, we observe additional stalling when the first arginine residue is positioned in the P site. We are not entirely certain why stalling occurs at both the first and second R codons in our MRLR templates. It is important to emphasize that macrolide-induced stalling is not limited to only +X+ motifs and arrest can also occur at other sequence contexts, including so-called xRx motifs, as described in the genome-wide study by Davis *et al.* (2014)². In that work, macrolide-dependent stalling was occasionally observed at motifs ending in a C-terminal arginine, though such arrest was highly context-dependent and not universally present across all xRx sequences. The broader context of the nascent peptide, and potentially the mRNA, appear to be important determinants. In our experiments, ERY-dependent stalling at the first R codon occurs when the incoming A-site aminoacyl-tRNA delivers leucine (lanes 2 and 4), but not when it delivers cysteine (lane 6), suggesting that the identity of the incoming amino acid also plays a role in modulating arrest efficiency, possibly by influencing the conformation of the nascent peptide within the tunnel.

A mechanistic explanation for the problematic nature of xRx motifs may lie in the structural challenges associated with nascent peptide translocation. Specifically, after peptide bond formation, the nascent chain must be repositioned within the ribosome during translocation. In the case of fMRL-peptidyl-tRNA, this repositioning requires the side chain of the penultimate arginine to undergo an $\sim 180^\circ$ twist, moving from a cavity it occupies in the pre-translocation state to a post-translocation site deeper in the exit tunnel. This rearrangement is already sterically demanding, but it becomes even more difficult in the presence of a macrolide. Our structural data show that the desosamine sugar of the drug overlaps with the post-translocation site that would normally accommodate the repositioned -1 Arg side chain. This steric conflict could hamper proper repositioning of the nascent chain, resulting in the ribosome becoming kinetically trapped in a post-peptidyl transfer but pre-translocation state, explaining the additional arrest at xRx motifs in certain contexts.

Although the mechanism underlying these xRx-related stall sites remains unclear and is unrelated to the +X+-specific mechanism that forms the main focus of our study, we appreciate the reviewer highlighting this important point. In response, we have added a description of these additional upstream stalling bands to the Results section and noted that macrolide-induced stalling can occur outside of the +X+ motif under specific sequence contexts.

4. In lane 2, there is also strong stalling at the second R residue in the MRLR sequence. The band intensity is comparable to the first R. How is this interpreted?

Response: As discussed in the previous comment, the stalling observed at the first R codon likely reflect context-dependent arrest on xRx motifs, influenced by both the sequence of the nascent peptide and the identity of the incoming aminoacyl-tRNA. Stalling at the second R codon appears to fall into the same category. A mechanistic explanation for this stalling can again be attributed to impaired peptide translocation within the macrolide-bound ribosome. At the second R codon, the ribosome likely contains fMRLRS-peptidyl-tRNA in the A site and may become trapped in a post-peptidyl transfer, pre-translocation state for the reasons discussed in our response to the previous comment. Notably, this stalling strictly depends on the presence of an arginine at position 4 of the nascent peptide. When R4 is replaced by lysine (as in lanes 4 and 6), translocation appears to proceed normally and no arrest at the second R codon is observed.

5. Lanes 2 and 4 (MRL) show stronger stalling than lane 6 (MRC). What does this suggest about the difference between leucine and cysteine at this position?

Response: The reviewer is correct that the intensity of stalling on MRCK sequence is weaker than on MRLR or MRLK. On the one hand, previous *in vitro* data have suggested that the identity of the ultimate residue of the nascent chain is largely dispensable for stalling at +X+ motifs (see ref. 38). However, genome-wide ribosome profiling studies conducted *in vivo* present a more nuanced view: the intensity of arrest at +X+ motifs does appear to depend on the nature of the residue in the central “X” position. Specifically, the strength of stalling generally increases with the size of the side chain of the X residue. This phenomenon aligns with the mechanistic framework described in our previous two responses. Following the same logic, we hypothesize that bulkier residues in the X position (such as leucine) may further restrict the conformational freedom of the penultimate Arg/Lys residue, potentially locking it into a stalling-competent geometry. In contrast, smaller or more flexible residues (such as cysteine)

in the X position may provide greater freedom for the penultimate residue to adopt alternative, stalling-incompetent conformations, thereby weakening the arrest signal. Thus, the reduced stalling observed in lane 6 (MRC) may result from decreased steric constraint imposed by the cysteine side chain, compared to leucine in MRL (lanes 2 and 4). Alternatively, we cannot rule out the contribution of differences at the mRNA level. The identity of the downstream codons (CGU in lane 2 vs. AAA in lane 6) may influence local mRNA secondary structure, which could modulate arrest efficiency.

6. *In the MR vs. MRL comparison, MR shows a more intense band at the first nucleotide, while MRL shows a stronger second-band signal. Does this pattern align with the proposed arrest mechanism?*

Response: In our experience, the distribution of stalling signals between the +1 and +2 codon positions often varies between experiments and likely reflects minor differences in experimental conditions, such as reverse transcription efficiencies or ribosome-tRNA dynamics. For this reason, we generally do not consider the relative intensities of the upper and lower bands to be mechanistically informative.

That said, based on prior studies and our own observations, the upper band in a toeprinting assay typically corresponds to ribosomes with an empty A site, while the lower band reflects ribosomes in which the A site is occupied by a tRNA. From this perspective, the stronger second-band signal seen in the MRL-stalling (lane 2) is entirely consistent with our structural model, in which the incoming Arg/Lys-tRNA is accommodated in the A site but adopts an unproductive conformation due to the displacement of the penultimate R/K residue into the A-site cleft.

In contrast, the stronger upper bands observed in stalling at the first and second R codons, is more difficult to interpret within the framework of our +X+ model. As discussed in our earlier responses, this arrest likely reflects an alternative type of stalling, associated with the xRx motif, whose mechanistic basis remains poorly defined. In the absence of a clear structural model for xRx-mediated arrest, it is difficult to determine whether the observed band pattern is consistent or inconsistent with expectations. The apparent A-site emptiness (as inferred from the dominant upper band) may reflect an early translocation intermediate or a spontaneously ratcheted ribosomal state, rather than a canonical stalled elongation complex. Importantly, this form of stalling is distinct from the +X+ motif-driven arrest that is the primary focus of our study.

7. *Notably, arrest induced by catch-codons also shows stronger first-band signals. Could the authors comment on these observations to further validate their model?*

Response: Please see our response to the previous comment. Indeed, in the case of catch-codon-induced arrest, the strong upper-band toeprint signal reflects ribosomes with an empty A site. Mechanistically, this is entirely expected: in these experiments, we use mupirocin to inhibit isoleucyl-tRNA synthetase, leading to depletion of charged isoleucyl-tRNA in the system. As a result, when the ribosome encounters an isoleucine codon in the A site, no aminoacyl-tRNA is available for delivery by EF-Tu, and the A site remains vacant. This outcome is fully consistent with our interpretation in the previous response, namely, that dominant upper bands in toeprinting assays often indicate ribosomes stalled with an empty A site. Thus, the behavior of ribosomes stalled at catch codons serves as an additional example supporting our general model for interpreting toeprint band patterns.

8. *In some structural figures, color differences between components are too subtle, making interpretation difficult. Please consider enhancing contrast to improve visual clarity.*

Response: Following this suggestion of the reviewer, we have revised several figures to enhance color contrast between key components for improved clarity and ease of interpretation.

REFERENCES

1. Hedstrom, L. IMP dehydrogenase: structure, mechanism, and inhibition. *Chem. Rev.* **109**, 2903-2928 (2009).
2. Davis, A.R., Gohara, D.W. & Yap, M.N. Sequence selectivity of macrolide-induced translational attenuation. *Proc. Natl. Acad. Sci. USA* **111**, 15379-15384 (2014).

Response to Reviewer #4

Additional remarks to the authors:

1. *Figure 1c vs. Figure 2d: It seems like that the conformation of the A76 residue of the P-site tRNA in Figure 1c differs from that shown in Figure 2d (the deep blue model). Please carefully verify whether this discrepancy is real or the result of visualization/rendering differences. I suggest not use deep blue and show the N and O atoms in Figure 1c.*

Response: Indeed, when comparing the panels shown in Figures 1c and 2d, one might have the impression that the conformation of the P-site tRNA A76 residue appears somewhat different. We confirm that this visual discrepancy arises purely from the slightly different viewing angles used in the two figures. We have verified that both figures were rendered from exactly the same structural model. In the arrested complex, the 2'-OH of the A76 ribose in its C3'-endo pucker conformation merges visually with the ribose ring, which may have contributed to the reviewer's impression. Following the reviewer's helpful suggestion, we now display the 2'-OH group in red in Figure 1c (as well as in Supplementary Figure 3), which should alleviate this potential confusion. We have also displayed other oxygens in red and nitrogens in blue.

At the same time, we would like to note that in Figure 2d we intentionally compare the arrested and non-arrested states, and in this case, the ribose puckers are in fact distinct. Specifically, in the non-arrested state, the A76 adopts a C2'-endo pucker conformation, corresponding to a transpeptidation-competent state of the PTC. In contrast, in the macrolide-arrested state, the A76 ribose assumes a C3'-endo pucker, which is unable to sustain the H-bonding network required for deprotonation of the attacking α -amino group and maintain the proton relay ("proton wire"). This conformational difference highlights the functionally compromised state of the PTC in the arrested complex. Although we are not certain whether this structural distinction was the basis of the reviewer's comment, we are grateful for the opportunity to clarify it here. Moreover, we now emphasize in the manuscript the functional implications of the C3'-endo pucker of A76 in the arrested complex. We are grateful to the reviewer for prompting this important clarification.

2. *Analysis of +X+ stalling in Figure S7: The intent of Figure S7 is to demonstrate that the stalling pattern for the +X+ motif is similar between ERY and TEL treatment. Instead of presenting four representative regions, I suggest performing a quantitative comparison, such as calculating the correlation coefficient between the two datasets. Furthermore, approximately half of the +X+ sites appear to exhibit no detectable stalling, which raises the question of whether additional sequence or structural context is required to induce stalling. Given the apparent overall correlation between ERY- and TEL-induced stalling at +X+ sites, it would be valuable to further analyze or discuss whether the presence of a +X+ motif alone is sufficient for stalling, or whether other factors modulate this effect.*

Response: Excellent point and suggestion! Following the recommendation, we have now performed a quantitative comparison of ERY- and TEL-induced stalling across all identified +X+ motifs in the published Ribo-seq dataset. As expected, this analysis revealed a positive correlation between the two datasets ($R = 0.652$; see revised Supplementary Fig. 7), consistent with our structural finding that the desosamine moiety common to both ERY and TEL plays the central role in inducing +X+-

mediated stalling. We have revised the text in the Results section accordingly to report this correlation.

At the same time, we fully agree with the reviewer that not all +X+ motifs serve as efficient stalling sites. In fact, our analysis shows that about 22% of the +X+ sites (730 out of 3308) exhibit no detectable stalling in the Ribo-seq data (Supplementary Fig. 7, grey). We now explicitly discuss this in the Results section, emphasizing that +X+ should be viewed as a core determinant rather than a complete stalling motif. As we show using the ErmDL leader peptide as an example (citing Beckert *et al.*), upstream residues can strongly modulate the stalling outcome, with specific substitutions eliminating otherwise robust +X+-dependent arrest. Consistent with this, our structural and biochemical data suggest that particular combinations of upstream or penultimate (-1) and incoming (+1) residues can even displace a bound macrolide from the NPET, providing a mechanistic rationale for why certain +X+ motifs exhibit weak or no stalling.

Taken together, these new analyses and discussions underscore the reviewer's point: while the +X+ triplet is the minimal and most robust motif for macrolide-induced stalling, additional sequence context modulates the efficiency and, in some cases, determines whether stalling occurs at all. We thank the reviewer for prompting us to strengthen this important aspect of the manuscript.

3. *The authors stated that the reported resolutions are based on an $I/\sigma(I)$ threshold of 1.0. However, Table S1 indicates that the $I/\sigma(I)$ values for some outer resolution shells range between 0.82 and 0.88, which appears inconsistent with the stated threshold.*

Response: We thank the reviewer for this careful observation and fully agree with the comment. The discrepancy arose because, during the initial X-ray data processing, we typically rounded the resolution to the nearest 0.05Å during scaling. To address this issue, we have now re-scaled the crystallographic data for the two datasets in question (complexes 2 and 3) to resolutions at which $I/\sigma I$ equals exactly 1.0. Specifically, dataset 2 has been updated from 2.80Å to 2.83Å, and dataset 3 from 2.60Å to 2.65Å. Consequently, some parameters in the upper portion of Supplementary Table 1 have changed slightly, and the revised resolution values are now consistently reported in Supplementary Table 1, Figure 2, and Supplementary Figure 3.