

Anti-phage defense mechanism involving phage-encoded DNA binding protein and bacterial reverse transcriptase DRT4

Corresponding Author: Dr Zengqin Deng

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)

In this manuscript by Wang and Wu et al, the authors investigate the structure and molecular mechanism of an antiphage defense-related reverse transcriptase (DRT4). They demonstrate that DRT4 is a single-gene antiphage defense system that synthesizes random-sequence single-stranded DNA in a template-independent manner. Through point mutagenesis, the authors establish that the catalytic DNA polymerization activity is essential for antiphage function, with a conserved catalytic tyrosine residue responsible for the priming reaction. Using a combination of biochemical, structural, and functional assays, the authors show that DRT4 assembles into a dimer of trimers—an overall hexameric oligomer that is covalently linked to ssDNA. They identify key residues at the interaction interfaces among the protomers, as well as those involved in DNA binding. Mutagenesis of these residues leads to a loss of function, indicating that higher-order oligomerization is indispensable for antiphage activity. Through RNA-Seq, co-expression, and dilution spotting assays, the authors determine that DRT4 detects phage infection by sensing the ORF55 phage protein. They propose that ORF55 binds to the 3' end of the ssDNA and protects it from degradation by the host exonuclease ExoI—speculating a regulatory mechanism.

Based on these findings, the authors propose an overall model: in the absence of phage infection, DRT4 constitutively synthesizes ssDNA in a template-independent manner. The length of this ssDNA is limited by degradation via 3'–5' exonucleases in normal conditions. Upon phage infection, ORF55 binds to and protects the 3' end of the ssDNA, allowing its elongation. Accumulation of elongated ssDNA is toxic to the host cell, thereby conferring broad antiphage protection to the bacterial population through an abortive infection mechanism.

In my opinion, this study is both timely and compelling, and its findings will likely be of broad interest. However, several claims made in the manuscript are speculative and lack rigorous experimental validation. Additionally, I found inconsistencies in the methodology and several missing details throughout the manuscript that are crucial for the reproducibility. I have outlined my concerns and provided several suggestions below, which I believe need to be addressed for this work to be considered for publication in Nature Communications. I hope that the authors find these suggestions useful.

Major points:

The authors propose that the accumulation of long ssDNA leads to toxic effects in bacteria. However, there is no data demonstrating that ssDNA length increases upon phage infection. As it stands, this hypothesis appears to be entirely based on in vitro observations of relatively longer ssDNA length. One way to test this would be to perform immunoblotting of DRT4 (possibly fused with an epitope tag) in cell lysates with and without phage infection, ideally at early time points. Experimental validation of increased longer ssDNA accumulation during phage infection is crucial to rule out alternative mechanisms contributing to cellular toxicity.

Related-- The mechanism by which ssDNA accumulation causes toxicity remains unclear. Can the authors elaborate on this further?

Through structural modeling with the PriA homolog and biochemical assays, the authors demonstrate that ORF55 binds ssDNA. Have they tested whether ORF55 forms a stable complex with the DRT4–ssDNA complex? Have they attempted

structural modeling (maybe through AlphaFold3) to determine if DRT4 mediates additional interactions with ORF55? Moreover, the functional role of ORF55 is not discussed. Is this protein essential for phage replication?

The RNA-seq data used to identify ORF55 is not provided with the manuscript. One would expect this dataset to be included as a supplementary file. Additionally, there is no mention of RNA-seq data deposition in a public repository, which is mandatory according to the reporting summary guidelines signed by authors.

In the cryo-EM structure determination, it is unclear whether all protomers participate in ssDNA synthesis or if polymerization activity is restricted to a subset. Before applying D3 symmetry, did the authors verify that the particles were truly symmetric?

Have the authors tested the potential interference of ORF55 in ssDNA synthesis by DRT4? It is possible that ORF55 binding could also inhibit the polymerization activity of DRT4. The authors could consider performing such an assay to further support their model.

The Results section (line 106) mentions that the MG1655 strain was used for plaque assays, but the Methods section (line 548) states that the BL21 (DE3) strain was used. Please clarify and correct this inconsistency.

The plaque assays in Figure S1e show no discernible difference for T6 phage. The authors may consider performing additional dilutions to determine if there is any measurable protection.

Figure 3c: The polymerase activity of the MBP–DRT4 (Y125F) fusion protein is tested however the wild type DRT4 protein (used as a control) is not MBP tagged. It is unclear whether the MBP tag affects DRT4's enzymatic function. Can the authors comment on this or provide experimental evidence confirming that the MBP fusion does not interfere with polymerase activity?

Minor points:

Line 413–415: Please provide more details on how protein expression was induced.

Lines 479–482: The induction protocol is poorly described. Please specify the duration of post-induction growth and the temperature used

Please comment on the residues located in the disallowed region (0.2%) of the model.

Show the catalytic YIDD motif residues as sticks in Figure 2c.

For Figures 4a and 4b, specify the number of replicates performed. Define “n” and include error bars with individual data points.

In Figure 4c, specify the dye used and mention it in the figure legend.

In the Figure 6 legend, “Hexmeric” should be corrected to “hexameric,” and “toxicity” should be revised to “toxic.”

Line 932: Please provide the PDB ID used for Figure 5a.

Lines 496–497: Provide citations for the software tools SEDFIT and SADPHAT.

The authors may consider rearranging figure panels to match the order in which the data are discussed in the manuscript. For instance, Figure 1f could be moved to Figure 1a, as it represents the first result discussed in the Results section.

Reviewer #2

(Remarks to the Author)

This study reveals how bacterial reverse transcriptase DRT4 synthesizes random ssDNA via protein priming to defend against phages. The authors show phage protein ORF55 protects ssDNA from degradation, triggering toxic accumulation and cell death. The conserved mechanism extends to DRT6, demonstrating a new anti-phage strategy where phage proteins activate host suicide. This is a well-presented study that provides important new insights into the bacterial reverse transcriptase. I recommend publish of this work if the following points are addressed.

Key points:

1. The presence of two nucleic acid peaks following the main protein peak in the SEC profile (Figure S1a) suggests potential dissociation of protein-nucleic acid complexes during chromatography. Could the authors please address whether these peaks represent genuine dissociation products, or they might reflect contamination from unbound nucleic acids?
2. The SEC profile in Figure 3c displays multiple peaks for the MBP-Y125F fusion construct, raising two key concerns that require clarification. First, the molecular identity of the secondary peaks needs to be determined, as they could represent degradation products, alternative oligomeric states, or MBP-free protein. More importantly, since activity assays were performed without removing the MBP tag, it remains unclear whether this large fusion tag (~40 kDa) might interfere with critical functional aspects such as protein oligomerization (known to be essential for DRT4 activity based on Figure 2) or

nucleotide binding (particularly concerning given Y125's proximity to the active site).

Minor points:

1. P4 line 108: The figure references would be clearer if presented in their order of appearance (currently citing Figure 1f before S1e).
2. P5 line 113: The 1.9 AU peak height in Figure 1a seems exceptionally intense - could the authors comment on whether this affects resolution between different oligomeric states? An expanded view of the peak region might help evaluate potential peak overlap.
3. P5 line 136: The authors state that "purified DRT4 is only associated with short oligonucleotides", but no supporting data are presented to demonstrate.
4. Several figures presenting phage challenge assays (e.g., Figure 1f and S1e) appear to show the phage titer dilutions in reverse order. Please check the dilution order.
5. The phage challenge assays in Figure 3b currently lack explicit indication of the phage titer/dilution series used in the spot assays.

Reviewer #3

(Remarks to the Author)

The manuscript by Wang et al shows that the defence system DRT4 acts as a template-independent ssDNA polymerase and is activated by a phage-encoded DNA-binding protein, ORF55. Using high-resolution cryo-EM, the authors could capture multiple functional states of DRT4, such as ssDNA engagement, metal ion coordination, and a priming mechanism involving a specific tyrosine residue. The study also hints at a broader activation paradigm by showing that ORF55 can activate DRT6, a related DRT protein. This is a compelling and well-executed piece of work that pushes forward our understanding of defence-associated RTs. That said, several conceptual and mechanistic points remain unclear, and the authors should address these before the manuscript can be considered for publication.

1. The authors propose that ORF55 protects DRT4-generated ssDNA from degradation, allowing it to build up to toxic levels and trigger cell death. But this key part of the mechanism is not directly shown. There is no in vivo measurement of ssDNA accumulation, such as qPCR or fluorescent oligo labelling, and no clear demonstration that stabilising the ssDNA actually causes cell death. Without that, the idea remains a plausible but untested hypothesis. The authors should either provide experimental evidence or make it clear that this is still speculative.
2. The co-expression data make a strong case that ORF55 activates DRT4, but since this is based on overexpression, there is always a risk of artefacts, and that should be noted. More importantly, it is still not clear how ORF55 activates DRT4. Does it just bind and shield the ssDNA from degradation, or is it doing something more active, like altering DRT4 conformation or recruitment? It would also help to know whether DNA-binding-deficient ORF55 mutants still interact with DRT4 or co-localise with its products.
3. The authors convincingly demonstrate that DRT4 can synthesise random-sequence ssDNA in vitro, primed by Tyr125. But in vivo, DRT4 appears to be associated only with short DNA fragments. This discrepancy is noted but not explored. What do DRT4-derived ssDNA products look like in cells? Do ORF55 or phage infection increase their stability or length?
4. The cryo-EM work is elegant, and the structural models are of high quality. However, the architecture of DRT4 is similar to previously described AbiK structures. The authors should clarify what is novel here: is it the oligomerisation, the mode of substrate binding, or the activation mechanism? Also, while the α Rep domain is said to replace the thumb subdomain of canonical RTs, its role in DRT4 function is not tested. Does it contribute to trimerisation, substrate recognition, or something else?
5. The finding that ORF55 also activates DRT6 is interesting, but currently underdeveloped. If the authors want to make the case for a conserved activation mechanism, more evidence is needed, for example conservation of key residues (Tyr125), oligomeric state, or DNA-binding features. As it stands, the mention of DRT6 would be better placed in the Discussion unless supported by additional data.
6. Many of the figure legends, especially for the structural data, are overly dense and hard to follow. Residues, domains, and interaction surfaces should be labelled more clearly in both the figures and the legends to aid reader interpretation.

Reviewer #4

(Remarks to the Author)

Defense associated reverse transcriptase (DRT) are recently identified RT from prokaryotic systems that may function in bacterial immune response against phage infection. While DRT2 structure, mechanism and antiphage defense has been studied (Wilkinson, Zhang, 2024), most of DRTs are relatively unknown. In this report, Wang, Deng and colleagues characterized biochemical activity, phage defense and structure of DRT4. They determined high-resolution structure of DRT4 in dimer or trimer form with covalently associated DNA, revealing key residues for catalysis. In addition, they identified phage ORF55 single-stranded DNA binding protein as a regulator of DRT4 for initiating DRT defense. The study provides novel insight into DRT-mediated anti-phage defense system. I have several major comments:

1. The author showed metal ion dependent polymerase activity of DRT4. Specifically, they found Mn^{2+} can support robust template-independent DNA synthesis, while Mg^{2+} cannot. Mn^{2+} is known to stimulate error-prone DNA synthesis activities of many DNA polymerase and RTs due to its relaxed coordination requirement. However, the physiological concentration of Mn^{2+} in bacteria is generally low (~ 10 μM), much lower than the 5 mM used. To prove that Mn^{2+} -supported activity is relevant, the author needs to show that DRT4 can work with low Mn^{2+} . Otherwise, DRT4 may require other co-factors or

metal ions for its function.

2. The author showed that T5 ORF55 can stimulate DRT4 defense, and propose that the ORF55 can protect the nonspecific sequence from degradation. However, I have several comments with this hypothesis. First, MST assays in Fig 5 shows a very high affinity of ORF55 to ssDNA, ~8 nM, however, half saturation according to the curve is ~100 nM. The author needs to provide details for data fitting.

In addition, according to the method and Fig. S7d, 2 uM ORF55 shows partial shift and 100 uM ORF55 shows complete shift, which indicates relative low affinity binding of ORF55. Consistently, very high concentrations (> 10 uM) of ORF55 are needed to fully protect the ssDNA in vitro in Fig. S7e. The authors may need to perform careful EMSA titrations and compare it with MST to accurately assign binding affinity.

In addition, is there specific protein-protein interactions between ORF55 and DRT4? Will ORF55 stimulate DRT4 synthesis, especially with physiological relevant Mg²⁺?

3. DRT2 is known to associated with ncRNA. However, ncRNA is not mentioned for DRR4, which is a miss. In addition, diagram of genomic locus of DRT4 will be helpful. Do DRT4 has associated RNA or protein for its antiphage defense?

Minor comments.

Fig 3c, the label should be MBP-DRT4, not MPB-DRT4.

Fig S1d, the band for dCTP is particularly weak. Is it due to staining or the low product yield with dCTP. If the later case, it is in contrast to the conclusion that DRT4 has no dNTP preference.

It would be helpful to provide proteins concentrations for titration assays directly in figure legends, Fig 5e, S7d,e.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This reviewer is pleased to see the revised version of the manuscript, where all the major and minor comments have been addressed. Therefore, I have no further comments. In my opinion, the current version of the manuscript is suitable for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)

The author addressed my concerns, I recommend publication of this work

Reviewer #3

(Remarks to the Author)

The authors have performed additional experiments and provided additional discussion, having addressed all concerns raised previously. I believe the work is now ready to be published.

Reviewer #4

(Remarks to the Author)

With new experiments and discussions, the authors have nicely addressed all my comments.

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We thank the reviewers for their positive and insightful comments, which are invaluable in improving our manuscript. We are pleased that the reviewers recognize our work as a timely and compelling study that is likely to be of broad interest. Below we have addressed the specific points raised by the reviewers and amended our manuscript accordingly. The revisions have been marked in blue font in the revised manuscript. We hope the reviewers find it suitable for publication.

Reviewer #1 (Remarks to the Author):

In this manuscript by Wang and Wu et al, the authors investigate the structure and molecular mechanism of an antiphage defense-related reverse transcriptase (DRT4). They demonstrate that DRT4 is a single-gene antiphage defense system that synthesizes random-sequence single-stranded DNA in a template-independent manner. Through point mutagenesis, the authors establish that the catalytic DNA polymerization activity is essential for antiphage function, with a conserved catalytic tyrosine residue responsible for the priming reaction. Using a combination of biochemical, structural, and functional assays, the authors show that DRT4 assembles into a dimer of trimers—an overall hexameric oligomer that is covalently linked to ssDNA. They identify key residues at the interaction interfaces among the protomers, as well as those involved in DNA binding. Mutagenesis of these residues leads to a loss of function, indicating that higher-order oligomerization is indispensable for antiphage activity. Through RNA-Seq, co-expression, and dilution spotting assays, the authors determine that DRT4 detects phage infection by sensing the ORF55 phage protein. They propose that ORF55 binds to the 3' end of the ssDNA and protects it from degradation by the host exonuclease ExoI—speculating a regulatory mechanism.

Based on these findings, the authors propose an overall model: in the absence of phage infection, DRT4 constitutively synthesizes ssDNA in a template-independent manner. The length of this ssDNA is limited by degradation via 3'–5' exonucleases in normal conditions. Upon phage infection, ORF55 binds to and protects the 3' end of the ssDNA, allowing its elongation. Accumulation of elongated ssDNA is toxic to the host cell, thereby conferring broad antiphage protection to the bacterial population through an abortive infection mechanism.

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my concerns and provided several suggestions below, which I believe need to be addressed for this work to be considered for publication in Nature Communications. I hope that the authors find these suggestions useful.

Major points:

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Related-- The mechanism by which ssDNA accumulation causes toxicity remains unclear. Can the authors elaborate on this further?

Thank the reviewer for this great suggestion. We performed immunoblotting of DRT4 with an N-terminal Flag-tag in cell lysates during phage infection. A high-molecular-weight band appeared after infection and became progressively stronger as the infection advanced (**Fig. R1**). This band was not observed in the catalytic-dead DRT4 mutant following phage infection. Notably, the band migrated above 250 kDa, larger than the DRT4-ssDNA covalent complex observed in the in vitro polymerization assay (**Fig. 1e**). Unidentified factors may enhance DRT4 processivity in vivo. Immunoblotting further revealed that DRT4 protein levels remained unchanged during phage infection, indicating that the DRT4 system is not regulated at the level of protein expression. Collectively, these results indicate that phage infection promotes the accumulation of longer ssDNA species.

It will be a high priority of our future work to elucidate the mechanism by which the longer ssDNA accumulation leads to cellular toxicity. In the Discussion section, we have outlined several possible mechanisms linking ssDNA accumulation to cell death.

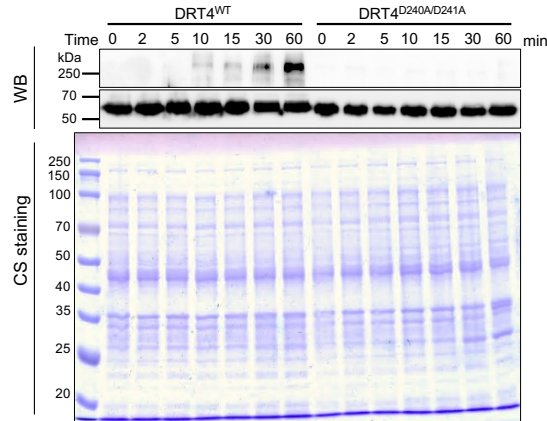


Fig. R1 Western blot (WB) analysis of DRT4 protein levels at different time points after phage infection (manuscript Fig. 6b). *E. coli* BL21(DE3) cells expressing Flag-tagged DRT4 or its mutant were infected with phage T5 at an MOI of 5, and DRT4 protein levels were monitored over time. In cells expressing wild-type DRT4, a high-molecular-weight band appeared after infection and intensified as the infection progressed, whereas no such band was observed in the catalytic-dead mutant.

2a. Through structural modeling with the PriA homolog and biochemical assays, the authors demonstrate that ORF55 binds ssDNA. Have they tested whether ORF55 forms a stable complex with the DRT4–ssDNA complex? Have they attempted structural modeling (maybe through AlphaFold3) to determine if DRT4 mediates additional interactions with ORF55?

We performed an MBP pull-down assay to test whether and how ORF55 forms a stable complex with the DRT4–ssDNA complex. As shown in **Fig. R2**, the wild-type DRT4 covalently associated with short ssDNA, as well as the wild-type DRT4 extended with dNTPs to generate longer ssDNA, both form stable complexes with ORF55. In contrast, the ORF55 Y18A mutant, which exhibits reduced ssDNA binding ability as determined by EMSA and is not toxic to cells when coexpressed with DRT4, showed diminished association with DRT4. Moreover, the catalytically inactive D240A/D241A mutant, which does not covalently link ssDNA, failed to interact with ORF55. Together, these results suggest that ssDNA mediates the interaction between ORF55 and DRT4, rather than ORF55 directly associating with DRT4 itself.

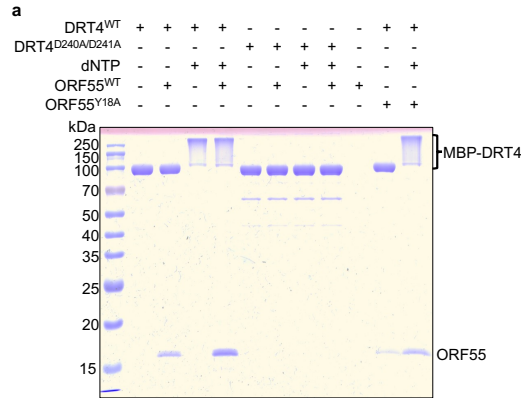


Fig. R2 ssDNA synthesized by DRT4 mediates the DRT4-ORF55 interaction (manuscript Fig. 6a). In vitro MBP pull-down of ORF55 by MBP-tagged DRT4 variants. MBP-tagged DRT4 proteins (wild-type and D240AD241A mutant) preincubated with or without dNTPs were used as baits. ORF55 and its Y18A mutant were used as prey. Two faint bands observed between 70 kDa and 40 kDa likely correspond to degraded DRT4 and the MBP tag.

2b. Moreover, the functional role of ORF55 is not discussed. Is this protein essential for phage replication?

Currently, no functional studies of ORF55 have been reported. The role of ORF55 in the phage T5 life cycle remains unknown. However, the *orf55* gene is located within a deletable region of the phage T5 genome, suggesting that it is non-essential for phage T5¹. We have added this information in the revised manuscript.

3. The RNA-seq data used to identify ORF55 is not provided with the manuscript. One would expect this dataset to be included as a supplementary file. Additionally, there is no mention of RNA-seq data deposition in a public repository, which is mandatory according to the reporting summary guidelines signed by authors.

The RNA-seq data used to identify ORF55 have been deposited in the SRA database under accession code PRJNA1280264. These data are also provided as the source data of supplementary Fig 7a in the revised manuscript.

4. In the cryo-EM structure determination, it is unclear whether all protomers participate in ssDNA synthesis or if polymerization activity is restricted to a subset. Before applying D3 symmetry, did the authors verify that the particles were truly symmetric?

We performed 3D reconstruction without symmetry imposed (C1 symmetry), resulting in a cryo-EM map with an overall resolution of 2.65 Å, which is lower than the 2.27 Å resolution reconstruction obtained with D3 symmetry imposed. The hexameric DRT4 model fits well into the map, clearly showing each subunit along with corresponding densities for the ssDNA (**Fig. R3**). These results support that DRT4 is symmetric, with all protomers participating in ssDNA synthesis.

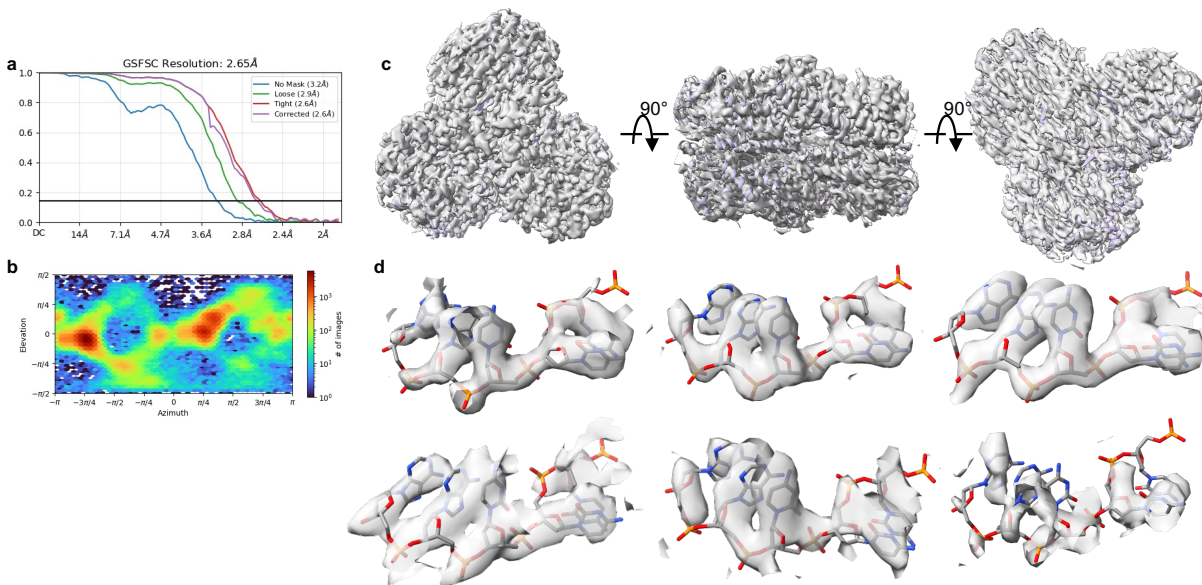


Figure R3. Cryo-EM reconstruction of DRT4 with C1 symmetry. **a** Fourier shell correlation (FSC) curves were calculated using two independent half maps, and resolution was estimated using the FSC=0.143 cutoff. **b** Particle distribution of DRT4. **c** Cryo-EM density map of DRT4 with the fitted atomic model. **d** Cryo-EM densities of ssDNA within six DRT4 protomers.

5. Have the authors tested the potential interference of ORF55 in ssDNA synthesis by DRT4? It is possible that ORF55 binding could also inhibit the polymerization activity of DRT4. The authors could consider performing such an assay to further support their model.

We thank the reviewer for this insightful suggestion. Our data support the notion that ORF55 protects the 3' end of DRT4-synthesized ssDNA from host exonuclease degradation. Because binding of the 3'-proximal sequence to the DNA-binding channel of DRT4, which positions the 3' end at the active site, is essential for its polymerization activity, dissociation of this sequence from the active site and DNA-binding channel followed by capture by ORF55, would be expected to inhibit further dNTP incorporation. To directly test this possibility, as suggested by

the reviewer, we performed in vitro DNA polymerase activity assays in the presence of increasing concentrations of ORF55. Consistent with our hypothesis, DRT4 polymerization activity was inhibited in an ORF55 concentration-dependent manner (**Fig. R4**).

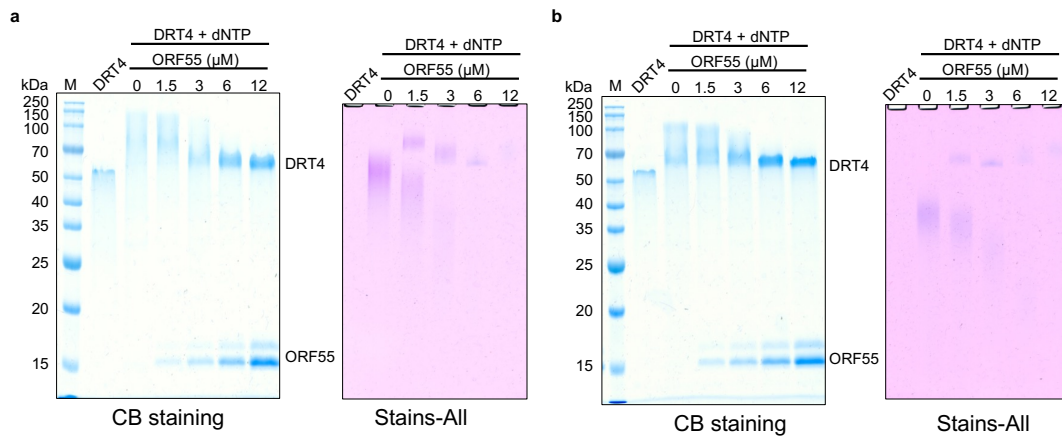


Fig. R4 Interference of ORF55 in ssDNA synthesis by DRT4 in vitro. **a** The reaction was carried out in a buffer containing 5 mM MnCl_2 . The products were directly analyzed by SDS-PAGE (left panel) or by denaturing urea-PAGE after proteinase K digestion (right panel). **b** The reaction was carried out in a buffer containing 5 mM MgCl_2 . The products were directly analyzed by SDS-PAGE (left panel) or by denaturing urea-PAGE after proteinase K digestion (right panel).

6. The Results section (line 106) mentions that the MG1655 strain was used for plaque assays, but the Methods section (line 548) states that the BL21 (DE3) strain was used. Please clarify and correct this inconsistency.

We apologize for the confusion. We would like to clarify that the plaque assays were conducted in both *E. coli* MG1655 and BL21(DE3) strains. While the DRT4 system functions effectively in both strains, we have chosen to present the experimental results obtained from BL21(DE3) for consistency. Accordingly, we have updated the main text to reflect the use of BL21(DE3) throughout.

7. The plaque assays in Figure S1e show no discernible difference for T6 phage. The authors may consider performing additional dilutions to determine if there is any measurable protection.

As suggested by the reviewer, we repeated the experiment with additional dilutions, which revealed a clear difference for phage T6, demonstrating that the DRT4 system confers protection against phage T6 (**Fig. R5**). The revised manuscript includes this updated figure.

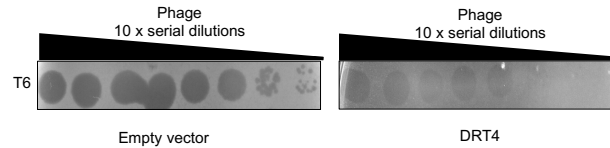


Fig. R5 Plaque formation of phage T6 on cells expressing empty vector or DRT4.

8. Figure 3c: The polymerase activity of the MBP–DRT4 (Y125F) fusion protein is tested however the wild type DRT4 protein (used as a control) is not MBP tagged. It is unclear whether the MBP tag affects DRT4’s enzymatic function. Can the authors comment on this or provide experimental evidence confirming that the MBP fusion does not interfere with polymerase activity?

As suggested by the reviewer, we verified that the MBP tag does not affect DRT4’s function. Plaque assays showed that MBP-DRT4 retains anti-phage activity (**Fig. R6a**), and in vitro enzymatic assays confirmed that the MBP tag does not alter DRT4’s enzymatic function (**Fig. R6b**). The enzymatic activity results of MBP-tagged DRT4 have been included in the revised manuscript.

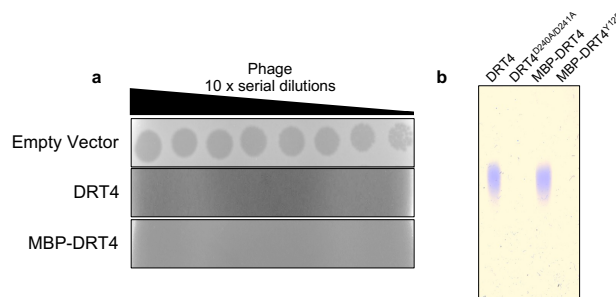


Fig. R6 Effect of MBP tag on the function of DRT4. a Plaque formation of phage T6 on cells expressing empty vector, DRT4, or MBP-DRT4. **b** Polymerization activity of DRT4 variants.

Minor points:

1. Line 413–415: Please provide more details on how protein expression was induced.

We apologize for the lack of clarity. DRT4 WT and mutants with an N-terminal His-tag (except R75A and Y125A) were expressed under the control of the native promoter, and thus did not require an inducer. This information has been added to the revised manuscript.

2. Lines 479–482: The induction protocol is poorly described. Please specify the duration of post-induction growth and the temperature used.

The detailed information of induction protocol has been included in the revised manuscript.

3. Please comment on the residues located in the disallowed region (0.2%) of the model.

In all the structures presented in this manuscript, residue I239, part of the catalytic YIDD motif, accounts for the 0.2% of residues in the disallowed region of the Ramachandran plot, exhibiting unusual backbone dihedral angles. Close inspection reveals that the cryo-EM density for this region is of high quality, supporting the accuracy of the model. The unfavorable conformation of I239 is stabilized by local hydrogen bonds, enabling the YIDD motif to form a tight turn that positions the catalytic aspartate residues (D240 and D241) for metal coordination and Y238 for DNA interaction. Such strained conformations are therefore functionally important (**Fig. R7**).

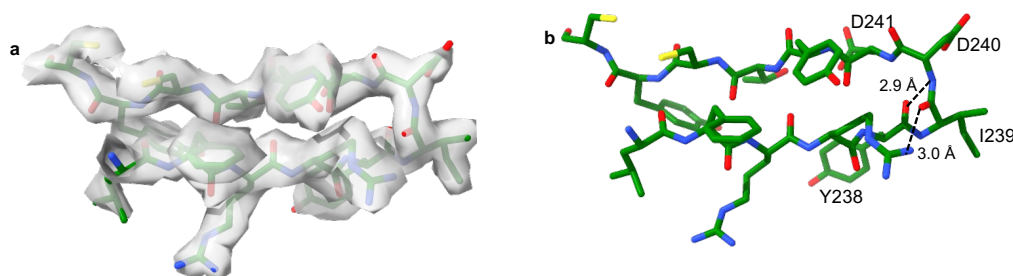


Fig. R7 Structure of the YIDD motif. **a** Cryo-EM map of the YIDD motif. **b** Detailed structure of the YIDD motif. Local H-bonds are shown.

4. Show the catalytic YIDD motif residues as sticks in Figure 2c.

The catalytic YIDD motif residues are shown as sticks in Figure 2c.

5. For Figures 4a and 4b, specify the number of replicates performed. Define “n” and include error bars with individual data points.

The number of replicates performed has been indicated in the figure legend and error bars with individual data points have been included in the figures.

6. In Figure 4c, specify the dye used and mention it in the figure legend.

The bacterial samples were stained with propidium iodide (PI). The information has been included in the figure legend.

7. In the Figure 6 legend, “Hexmeric” should be corrected to “hexameric,” and “toxicity” should be revised to “toxic.”

Thanks for pointing these typos out. The typos have been corrected.

8. Line 932: Please provide the PDB ID used for Figure 5a.

The PDB ID of *Klebsiella pneumoniae* PriA is presented both in the figure and main text.

9. Lines 496–497: Provide citations for the software tools SEDFIT and SADPHAT.

Citations are added.

10. The authors may consider rearranging figure panels to match the order in which the data are discussed in the manuscript. For instance, Figure 1f could be moved to Figure 1a, as it represents the first result discussed in the Results section.

Thanks for the suggestion. We have rearranged the figure panels of Figure 1 and Figure S1 to match the order in which the data are discussed in the manuscript.

Reviewer #2 (Remarks to the Author):

This study reveals how bacterial reverse transcriptase DRT4 synthesizes random ssDNA via protein priming to defend against phages. The authors show phage protein ORF55 protects ssDNA from degradation, triggering toxic accumulation and cell death. The conserved mechanism extends to DRT6, demonstrating a new anti-phage strategy where phage proteins activate host suicide. This is a well-presented study that provides important new insights into the bacterial reverse transcriptase. I recommend publish of this work if the following points are addressed.

Key points:

1. The presence of two nucleic acid peaks following the main protein peak in the SEC profile (Figure S1a) suggests potential dissociation of protein-nucleic acid complexes during chromatography. Could the authors please address whether: these peaks represent genuine dissociation products, or they might reflect contamination from unbound nucleic acids?

We have purified the catalytically dead mutant multiple times and found that while the main protein peak was consistent with pure DRT4 protein, the peak with a late elution volume varied from bench to bench (**Fig. R8**). We believe these peaks represent contamination from unbound nucleic acids and nonspecific proteins.

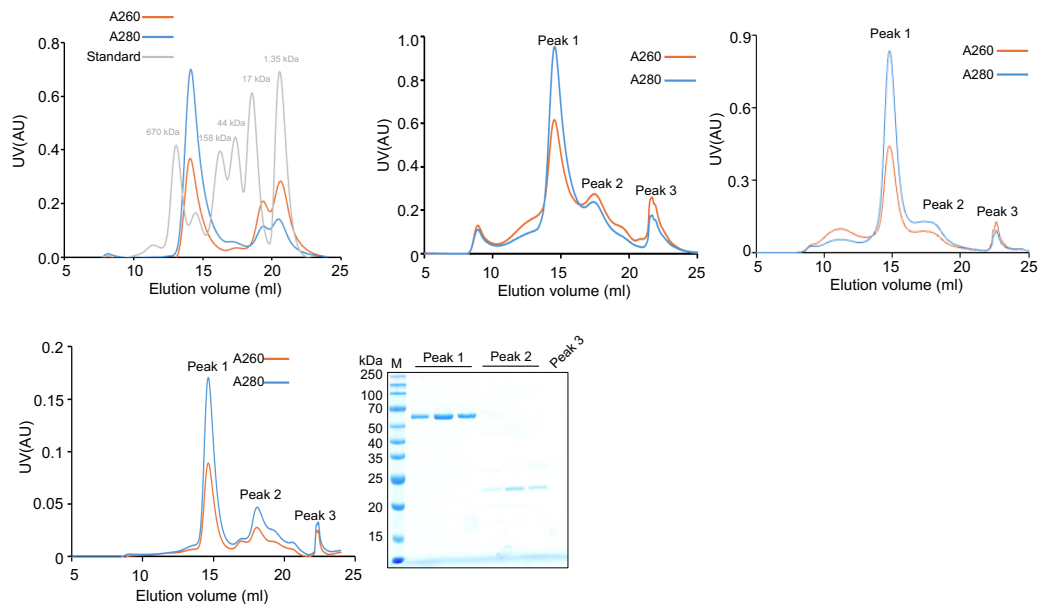


Fig. R8 SEC profiles of DRT4 D240A/D241A mutant with one representative SDS-PAGE gel.

2. The SEC profile in Figure 3c displays multiple peaks for the MBP-Y125F fusion construct, raising two key concerns that require clarification. First, the molecular identity of the secondary peaks needs to be determined, as they could represent degradation products, alternative oligomeric states, or MBP-free protein. More importantly, since activity assays were performed without removing the MBP tag, it remains unclear whether this large fusion tag (~40 kDa) might interfere with critical functional aspects such as protein oligomerization (known to be essential for DRT4 activity based on Figure 2) or nucleotide binding (particularly concerning given Y125's proximity to the active site).

To address the reviewer's concerns, we performed additional plaque assays and *in vitro* polymerization assays with MBP-DRT4. The plaque assays show that MBP-DRT4 retains anti-phage activity. Consistently, *in vitro* enzymatic assays confirm that the MBP tag does not alter DRT4's enzymatic function (**Please see the response to Major Point 8 of Reviewer 1 and Fig. R6**). In addition, we repeated the purification of MBP-tagged DRT4^{Y125F} and analyzed each fraction by SDS-PAGE (**Fig. R9**). The results show that peak 1 corresponds to highly pure oligomeric MBP-DRT4^{Y125F} protein, whereas the other peaks correspond to contaminating proteins. Taken together, these results indicate that the MBP tag does not interfere with the critical functions of DRT4.

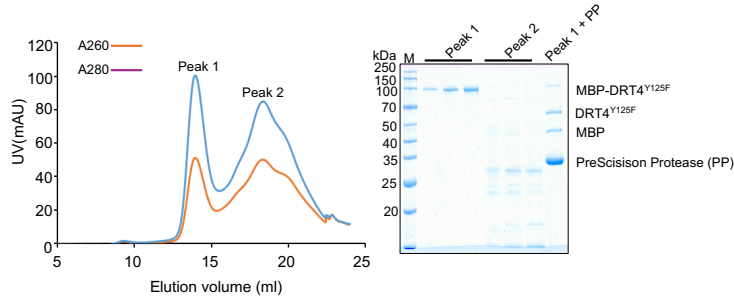


Fig. R9 (manuscript Fig. 3c) Purification result of MBP-DRT4^{Y125F} mutant.

Minor points:

1. P4 line 108: The figure references would be clearer if presented in their order of appearance (currently citing Figure 1f before S1e).

We have rearranged the figure panels of Figure 1 and Figure S1 to match the order in which the data are discussed in the manuscript.

2. P5 line 113: The 1.9 AU peak height in Figure 1a seems exceptionally intense - could the authors comment on whether this affects resolution between different oligomeric states? An expanded view of the peak region might help evaluate potential peak overlap.

In the revised manuscript, we included a new SEC profile obtained at a lower protein concentration, which shows a sharp and symmetric peak. We also present SEC profiles at various protein concentrations, all exhibiting similar peak shape (**Fig. R10**). Combined with cryo-EM observations, where only hexameric particles were detected, these results indicate that DRT4 exists exclusively as a hexamer.

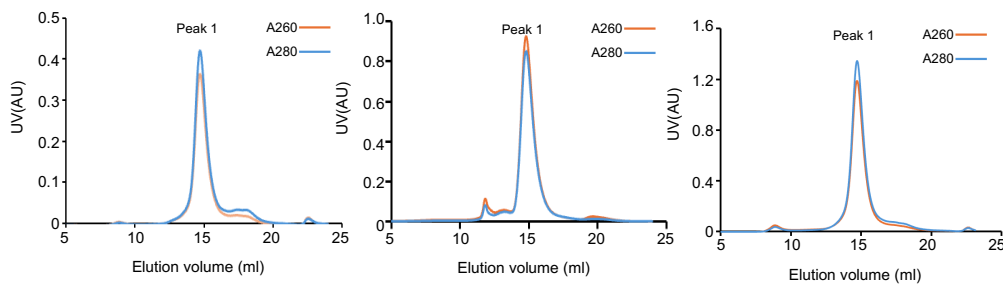


Fig. R10 SEC profiles of DRT4 at varying protein concentrations.

3. P5 line 136: The authors state that “purified DRT4 is only associated with short oligonucleotides”, but no supporting data are presented to demonstrate.

Two lines of evidence presented in the manuscript support this conclusion. First, analytical ultracentrifugation (AUC) shows that wild-type DRT4 forms a 418 kDa oligomer, substantially larger than its theoretical monomeric mass of 63.8 kDa. The catalytically inactive D240A/D241A mutant exhibits a slightly smaller oligomeric mass (~390 kDa), consistent with a loss of ~28 kDa per oligomer (~4.7 kDa per DRT4 protomer) (**Fig. 1c**). Second, structural comparison of the wild-type and D240A/D241A mutant reveals that the density of short oligonucleotides is traceable which is connected to residue Y125 (**Fig. 3a**).

4. Several figures presenting phage challenge assays (e.g., Figure 1f and S1e) appear to show the phage titer dilutions in reverse order. Please check the dilution order.

This has been corrected in the revised manuscript.

5. The phage challenge assays in Figure 3b currently lack explicit indication of the phage titer/dilution series used in the spot assays.

The information has been added in the figure and figure legend.

Reviewer #3 (Remarks to the Author):

The manuscript by Wang et al shows that the defence system DRT4 acts as a template-independent ssDNA polymerase and is activated by a phage-encoded DNA-binding protein, ORF55. Using high-resolution cryo-EM, the authors could capture multiple functional states of DRT4, such as ssDNA engagement, metal ion coordination, and a priming mechanism involving a specific tyrosine residue. The study also hints at a broader activation paradigm by showing that ORF55 can activate DRT6, a related DRT protein. This is a compelling and well-executed piece of work that pushes forward our understanding of defence-associated RTs. That said, several conceptual and mechanistic points remain unclear, and the authors should address these before the manuscript can be considered for publication.

1. The authors propose that ORF55 protects DRT4-generated ssDNA from degradation, allowing it to build up to toxic levels and trigger cell death. But this key part of the mechanism is not directly shown. There is no in vivo measurement of ssDNA accumulation, such as qPCR or fluorescent oligo labelling, and no clear demonstration that stabilising the ssDNA actually causes cell death. Without that, the idea remains a plausible but untested hypothesis. The authors should either provide experimental evidence or make it clear that this is still speculative.

In the revised manuscript, we performed immunoblotting of N-terminal Flag-tagged DRT4 in cell lysates following phage infection, as suggested by Reviewer 1. The results showed that covalent linkage of long ssDNA to DRT4 accumulates during infection (**please see our response to Major Point 1 from Reviewer 1 and Fig. R1**). However, although ssDNA accumulation is well documented to cause cellular toxicity under various cellular conditions and in several anti-phage systems²⁻⁶, we cannot directly demonstrate that ssDNA accumulation is the cause of cell death and reveal the underlying mechanism based on our current data. As the reviewer recommended, we have therefore softened the wording in the manuscript to emphasize that this interpretation remains speculative. Specifically, in the abstract we state, “**likely** resulting in toxic ssDNA accumulation that leads to cell death,” and in Figure 7 of the proposed model we have added a **question mark** after the sentence “Toxic ssDNA accumulation.”

2. The co-expression data make a strong case that ORF55 activates DRT4, but since this is based on overexpression, there is always a risk of artefacts, and that should be noted. More importantly, it is still not clear how ORF55 activates DRT4. Does it just bind and shield the ssDNA from degradation, or is it doing something more active, like altering DRT4 conformation or recruitment? It would also help to know whether DNA-binding-deficient ORF55 mutants still interact with DRT4 or co-localise with its products.

We thank the reviewer for raising this important point. We agree that results based on co-expression and overexpression may carry the risk of artefacts. To strengthen our conclusion that ORF55 activates the DRT4 system, we have provided extensive biochemical evidence showing that ORF55 activate DRT4 system by protecting the 3' end of DRT4-synthesized ssDNA from host exonuclease degradation. In the revised manuscript, we further demonstrate that ssDNA mediates the interaction between ORF55 and DRT4, as ORF55 does not directly associate with DRT4. Moreover, the ORF55-Y18A mutant, which fails to activate DRT4 in vivo, exhibited reduced DNA-binding capacity and diminished interaction with the DRT4-ssDNA complex (**Fig. R2 and Fig. R12c; Please see our response to Major Point 2a of Reviewer 1**). During the preparation of this manuscript, another group reported that this ORF55-mediated activation mechanism is also observed in another reverse transcriptase, DRT9, which synthesizes poly-dA in a ncRNA template-dependent manner³, underscoring the critical role of ORF55 in activating some DRT systems.

3. The authors convincingly demonstrate that DRT4 can synthesise random-sequence ssDNA in vitro, primed by Tyr125. But in vivo, DRT4 appears to be associated only with short DNA fragments. This discrepancy is noted but not explored. What do DRT4-derived ssDNA products look like in cells? Do ORF55 or phage infection increase their stability or length?

As the reviewer noted, we observed that the nucleic acids bound to the purified protein are much shorter than those synthesized in vitro. We hypothesized that this difference may arise because host factors either suppress the synthesis of long nucleic acids or degrade them after synthesis through host nucleases. To address this, we first demonstrated experimentally that ORF55 can bind to DRT4-synthesized nucleic acids in vitro and protect them from degradation by the *E. coli* 3'-5' exonuclease ExoI. Furthermore, when DRT4 was expressed alone in an *E. coli* ExoI-knockout strain ($\Delta sbcB$), we observed cell death, indicating that DRT4 is toxic in the absence of host nucleases. Finally, through infection experiments combined with Western blot analysis, we confirmed the accumulation of long nucleic acids (**Fig. R1, please see our reponse to Major Point 1 of Reviewer 1**).

Taken together, these results support the conclusion that, under normal conditions, DRT4-synthesized nucleic acids are degraded by host nucleases, explaining why only short nucleic acids are present in the purified protein. Upon phage infection, however, the phage protein ORF55 binds to the 3' end of DRT4 products and protects them from degradation.

4. The cryo-EM work is elegant, and the structural models are of high quality. However, the architecture of DRT4 is similar to previously described AbiK structures. The authors should clarify what is novel here: is it the oligomerisation, the mode of substrate binding, or the activation mechanism? Also, while the α Rep domain is said to replace the thumb subdomain of canonical RTs, its role in DRT4 function is not tested. Does it contribute to trimerisation, substrate recognition, or something else?

Despite the similar domain composition and overall organization of DRT4 and AbiK, notable structural differences exist. The RMSD between the DRT4 and AbiK protomers is 3.1 Å (over 303 pairs of C α atoms), while the RMSD of their RT domains is 1.5 Å (over 163 pairs of C α atoms). Even larger differences are observed in the α Rep domain, with an RMSD of 5.0 Å (over 141 pairs of C α atoms) (**Fig. S10b**). Their dimer-of-trimers organization also differs. In the DRT4 hexamer, the tilt angle between the upper and lower trimers is smaller ($\sim 10^\circ$) compared

with $\sim 60^\circ$ in AbiK. Furthermore, the primer amino acid in DRT4 is positioned in the palm region of the RT domain, whereas in AbiK it lies in the finger region, suggesting mechanistic differences in the initiation of nucleic acid synthesis (**Fig. S10a**). Finally, while AbiK requires nucleic acids to stabilize its hexameric assembly and predominantly exists as a monomer in the absence of nucleic acids, the DRT4 hexamer is intrinsically more stable and can form a stable hexamer even without ssDNA, as demonstrated by the catalytic-dead mutant, which assembles into a hexamer essentially identical to wild-type DRT4 except for the absence of ssDNA. We have added this information in the revised manuscript.

More importantly, in this study we resolved a high-resolution structure of DRT4 in complex with dNTP and catalytic metal ion, which reveals the coordination of the catalytic metal ion and substrate binding, providing mechanistic insights into its promiscuous polymerization activity.

The α Rep domain is essential for trimerization; as shown in **Fig. S4b**, it contributes to the formation of interface 1. Disruption of this interface abolishes the oligomeric state of DRT4, leading to the loss of both polymerization activity and anti-phage function (**Fig. S4c-e**). Two residues from the α Rep domain, Y337 and R301, also participate in ssDNA binding (**Fig. S5b**).

5. The finding that ORF55 also activates DRT6 is interesting, but currently underdeveloped. If the authors want to make the case for a conserved activation mechanism, more evidence is needed, for example conservation of key residues (Tyr125), oligomeric state, or DNA-binding features. As it stands, the mention of DRT6 would be better placed in the Discussion unless supported by additional data.

We fully agree with the reviewer that the finding that ORF55 also activates DRT6 is interesting but currently underdeveloped. As we have not yet determined the cryo-EM structure of DRT6 or identified its DNA-binding and protein-priming features. Therefore, we have placed this part in the discussion section.

6. Many of the figure legends, especially for the structural data, are overly dense and hard to follow. Residues, domains, and interaction surfaces should be labelled more clearly in both the figures and the legends to aid reader interpretation.

We have modified the labeling, figure order, and figure legends to improve clarity and facilitate easier interpretation for the reader.

Reviewer #4 (Remarks to the Author):

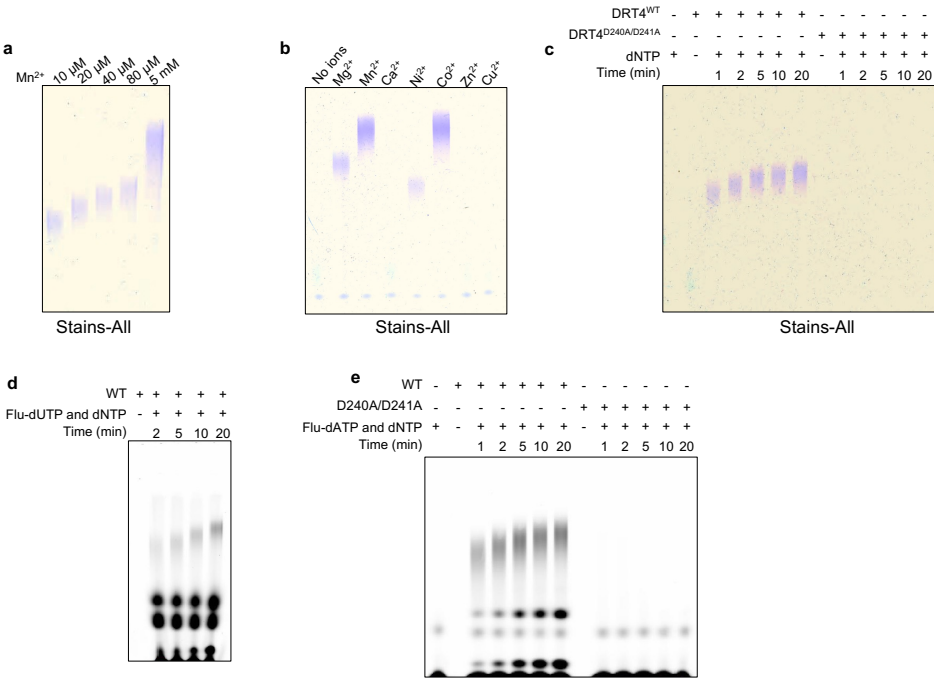
Defense associated reverse transcriptase (DRT) are recently identified RT from prokaryotic systems that may function in bacterial immune response against phage infection. While DRT2 structure, mechanism and antiphage defense has been studied (Wilkinson, Zhang, 2024), most of DRTs are relatively unknown. In this report, Wang, Deng and colleagues characterized biochemical activity, phage defense and structure of DRT4. They determined high-resolution structure of DRT4 in dimer or trimer form with covalently associated DNA, revealing key residues for catalysis. In addition, they identified phage ORF55 single-stranded DNA binding protein as a regulator of DRT4 for initiating DRT defense. The study provides novel insight into DRT-mediated anti-phage defense system. I have several major comments:

1. The author showed metal ion dependent polymerase activity of DRT4. Specifically, they found Mn^{2+} can support robust template-independent DNA synthesis, while Mg^{2+} cannot. Mn^{2+} is known to stimulate error-prone DNA synthesis activities of many DNA polymerase and RTs due to its relaxed coordination requirement. However, the physiological concentration of Mn^{2+} in bacteria is generally low (~ 10 μM), much lower than the 5 mM used. To prove that Mn^{2+} -supported activity is relevant, the author needs to show that DRT4 can work with low Mn^{2+} . Otherwise, DRT4 may require other co-factors or metal ions for its function.

We thank the reviewer for raising this concern. It is indeed correct that the intracellular concentration of Mn^{2+} in bacteria is typically in the micromolar range. We examined the enzymatic activity of DRT4 across varying Mn^{2+} concentrations and found that DRT4 is capable of synthesizing ssDNA even in the presence of micromolar Mn^{2+} , albeit with reduced processivity (**Fig. R11a**).

In our initial submission, we supplemented the reaction mixture with fluorescein-dUTP, expecting that incorporation of fluorescein-dUTP into ssDNA products would generate a stronger fluorescent signal, as previously applied in studies of AbiK⁷. Based on these experimental results, we initially concluded that DRT4 does not exhibit polymerization activity in the presence of Mg^{2+} . However, during revision we discovered that fluorescein-dUTP itself has relatively low incorporation efficiency under these conditions, resulting in weak signals that were only visible after overexposure (**Fig. R11d**). To address this, we repeated the assays using fluorescein-dATP, which confirmed that DRT4 is active in the presence of Mg^{2+} (**Fig. R11e**). We also performed assays with unmodified dNTPs and visualized the products using Stains-All (**Fig. R11b and c**).

The result clearly shows that DRT4 exhibits dependence on Mg^{2+} , Mn^{2+} , Ni^{2+} , or Co^{2+} for its polymerase activity, with no detectable catalytic activity in the presence of other tested divalent metal ions, including Ca^{2+} , Zn^{2+} , and Cu^{2+} .



1:1 equilibrium binding model without cooperativity, K_d and EC_{50} are related by the equation $EC_{50} = K_d + 0.5 \times [\text{target concentration}]$. Accordingly, when the target concentration is far below K_d , EC_{50} and K_d are nearly identical. However, when the target concentration approaches or exceeds K_d , EC_{50} can differ substantially from K_d . Because EC_{50} values are inherently dependent on experimental conditions, they cannot be directly compared across different assays.

In our experiments, to ensure a strong binding signal, the ORF55 concentration was set at 100 nM, which is higher than the K_d (ranging from 6 to 51 nM). This explains why the apparent half-saturation point in the binding curve deviates from the K_d . Details of the data fitting procedure used to calculate K_d are provided in the Methods section.

2b. In addition, according to the method and Fig. S7d, 2 μM ORF55 shows partial shift and 100 μM ORF55 shows complete shift, which indicates relative low affinity binding of ORF55. Consistently, very high concentrations ($> 10 \mu\text{M}$) of ORF55 are needed to fully protect the ssDNA in vitro in Fig. S7e. The authors may need to perform careful EMSA titrations and compare it with MST to accurately assign binding affinity.

We thank the reviewer for this suggestion. We performed careful EMSA titrations with 0.1 μM 5'-FAM-labeled 30-nt different ssDNA in the presence of increasing concentrations of ORF55. The results show that 0.25 μM ORF55 accounts for approximately half of the DNA binding, while 0.5 μM ORF55 results in nearly complete binding (**Fig. R12a**). We also performed EMSA titrations with the ORF55-Y18A mutant, which presumably disrupts the DNA-binding site. Consistent with this prediction, the EMSA results show that the Y18A mutation markedly reduces the DNA-binding ability of ORF55 (**Fig. R12b**). We also optimized the experimental condition of the protection experiments. The new result shows that 0.2-0.8 μM can fully protect the ssDNA (**Fig. R12c**).

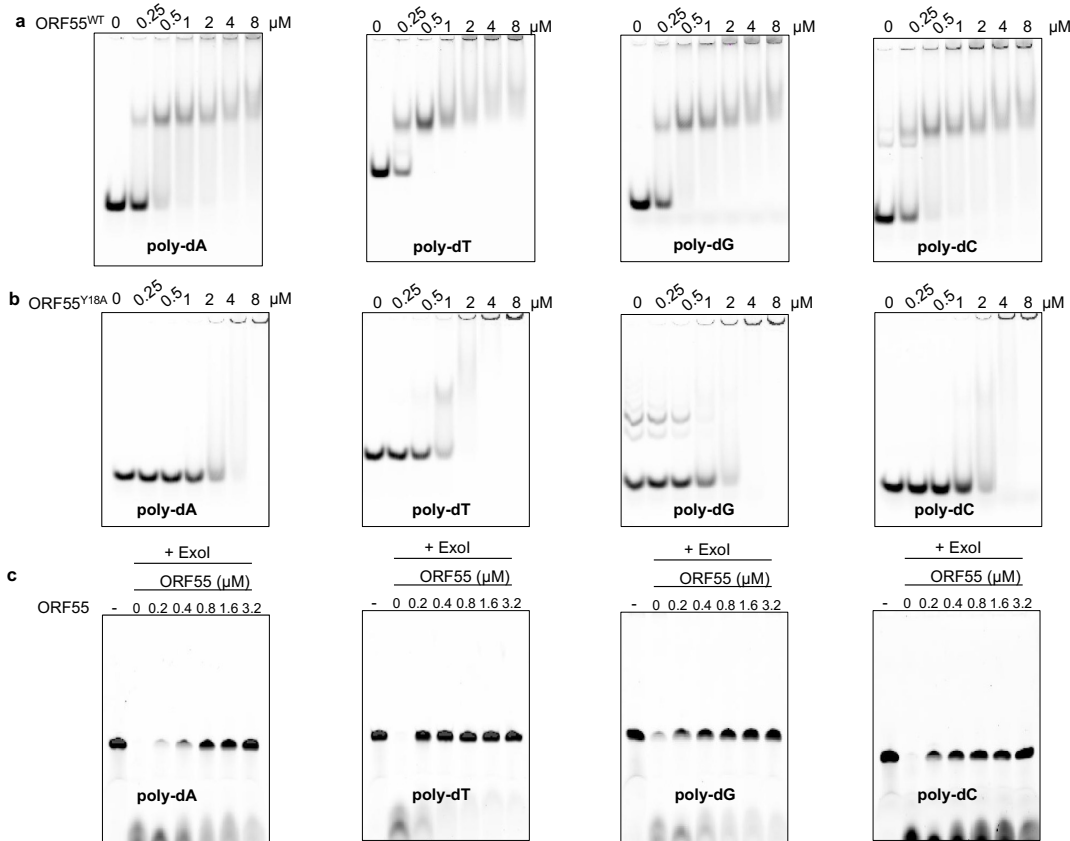


Fig. R12 ORF55 binds to single-stranded DNA (manuscript Supplementary Fig. 8). a EMSA of phage T5 ORF55 binding to different DNA homopolymers. Increasing concentrations of ORF55 were incubated with the 5'-FAM-labeled 30-nt ssDNA. b EMSA of phage T5 ORF55 Y18A mutant protein binding to different DNA homopolymers. c ExoI treatment of 5'-FAM-labeled 30-nt ssDNA in the presence of increasing concentrations of ORF55.

2c. In addition, is there specific protein-protein interactions between ORF55 and DRT4? Will ORF55 stimulate DRT4 synthesis, especially with physiological relevant Mg^{2+} ?

In the revised manuscript, we included MBP pull-down experiments demonstrating that ssDNA mediates the interaction between ORF55 and DRT4, whereas ORF55 does not directly associate with the DRT4 protein itself. **Please also refer to our response to Major Point 2a from Reviewer 1.** In the revised manuscript, we have included new data to show that DRT4 is active in the presence of 5 mM $MgCl_2$.

3. DRT2 is known to associated with ncRNA. However, ncRNA is not mentioned for DRR4, which is a miss. In addition, diagram of genomic locus of DRT4 will be helpful. Do DRT4 has associated RNA or protein for its antiphage defense?

Unlike DRT2, DRT4 does not require ncRNA or additional protein partners to execute its anti-phage function. This information has been added to the Results section ‘Functional characterization of DRT4’ of the revised manuscript.

Minor comments.

Fig 3c, the label should be MBP-DRT4, not MPB-DRT4.

The typo has been corrected.

Fig S1d, the band for dCTP is particularly weak. Is it due to staining or the low product yield with dCTP. If the later case, it is in contrast to the conclusion that DRT4 has no dNTP preference.

The poly-dCTP product appears pink when stained with stains-all dye, similar to the background color, which makes it seem faint. In the revised manuscript, we removed this figure due to the weak band. We used nucleotide composition analysis of the DNA products to demonstrate substrate preference of DRT4.

It would be helpful to provide proteins concentrations for titration assays directly in figure legends, Fig 5e, S7d,e.

The protein concentrations for titration assays have been provided in the revised figures.

- 1 Wang, J. *et al.* Complete genome sequence of bacteriophage T5. *Virology* **332**, 45-65 (2005).
- 2 Tan, J. M. J. *et al.* A DNA-gated molecular guard controls bacterial Hailong anti-phage defence. *Nature* **643**, 794-800 (2025).
- 3 Tang, S. *et al.* Protein-primed homopolymer synthesis by an antiviral reverse transcriptase. *Nature* (2025).
- 4 Erental, A., Kalderon, Z., Saada, A., Smith, Y. & Engelberg-Kulka, H. Apoptosis-Like Death, an Extreme SOS Response in Escherichia coli. *mBio* **5**, 10.1128/mbio.01426-01414 (2014).
- 5 Fonville, N. C., Bates, D., Hastings, P. J., Hanawalt, P. C. & Rosenberg, S. M. Role of RecA and the SOS Response in Thymineless Death in Escherichia coli. *PLOS Genetics* **6**, e1000865 (2010).
- 6 Song, X.-Y. *et al.* Bacterial reverse transcriptase synthesizes long poly-A-rich cDNA for antiphage defense. *Science* **0**, eads4639
- 7 Figiel, M. *et al.* Mechanism of protein-primed template-independent DNA synthesis by Abi polymerases. *Nucleic Acids Res* **50**, 10026-10040 (2022).