nature portfolio

Peer Review File

Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor

Reviewer #1 (Remarks to the Author):

The paper “Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor” by Lokareddy et al reports on a high resolution structure for phage DEV virions using cryo-EM methods. In addition, protein purification of two ejection proteins and lipid bilayer experiments show that these proteins form pores starting from the outer membrane of the host. Phage plating experiments are done to probe the primary receptor for interactions with the host. Overall most of the work is of high quality and this study is an important area of research as to date this is the first reported high resolution structure for an N4-like phage, which is a type of podophage quite different than other well described podophages (e.g. P22, Sf6, T7). Largely the paper is well written and enjoyable to read. However, there are some issues that detract from the work and some conclusions that are not well supported by the available data. These need to be addressed before the work is of suitable publication quality. I have broken down these points into major and minor issues and describe each below.

One overarching concern is uncertainty over the presence and/or copy number of several proteins. Specific examples are listed below. In general thorough biochemical analyses such as gels and mass spectrometry would help strengthen several hypotheses generated by the cryo-EM density.

In addition, currently the paper is targeting an audience that is phage-savvy and structure savvy, and more general information needs to be provided to make the work accessible to the broad readership of Nature Communications. I think it would be wise to move a portion of the Discussion (e.g. the first paragraph) upfront to the Introduction so the significance and importance is more obvious right away. In addition, some figures can be removed to highlight the more important points of the paper and not overwhelm the reader with details. Likewise the overabundance of supplementary data makes the reader flip between the main and supplemental section so often if becomes tedious.

I’d suggest the following figure removals:
1. Supplementary Figure 1: Other than panel A this figure does not add much. Also, this observation of partial and fully filled capsids takes on a substantial portion of the Results section and is the first thing presented, but this phenomenon is likely not physiologically relevant whatsoever.
2. Supplementary Figure 4: redundant with Fig 1 in the main text. What does this add?
3. Not sure what Figure 3 adds, especially given the uncertainly of the stoichiometry (see more on this point below).

MAJOR ISSUES:

• Abstract: The authors state ”We identified DEV ejection proteins and, unexpectedly, found that the giant DEV RNA polymerase, the hallmark of the Schitoviridae family, is an ejection protein.” This is not novel information as it has been well established that vRNAPs are ejected into the hosts for both T7 and N4. As far as labeling it an “ejection protein” and part of the motor…. the data shown for this claim within this paper are very weak/non existent (see below).
• Results: The authors state “Unfortunately, this density did not align with the capsid or portal axis and could not be improved. It likely belongs to the 3,398 amino acid vRNAP, as proposed for the phage N4 6.” There is really no evidence for this claim. What is the calculated volume of this

density? Does it correspond to the known size/copy number of the polymerase? While this could be the location, the authors have done a lot with symmetry and focused reconstruction methods that this could be entirely artifactual. A knockout would need to be studied and C1 reconstructions used for accurate comparisons.
• Results: The authors state "This suggests the cables do not play a structural role or stabilize the capsid from the inside but perhaps are involved in capsid assembly." This is a wildly inaccurate statement. Most if not all dsDNA phages first assemble to form procapsid shells and then the genome is packaged via a packaging terminase motor. How could the genome "cables" possibly be involved in assembly?
• Results: The authors state "The lack of physical constriction and the continuous DNA density inside the tail tube suggests that the DEV tail is sealed by a plug at the distal tip relative to the capsid, analogous to the tail needle of phage P22 21 and Sf6 20." The data shown in Supplementary data Figure 4B are not convincing that there is strong density here. Also, biochemical analysis would show if there was a protein positively identified in the mature virion that is not accounted for by the other assigned cryo-EM density.
• Results: I have trouble with the entire sub-section entitled "DEV long-tail fiber gp53 assembles to the tail via a 15:12 symmetry mismatch". This is very different than seen for N4 (Choi et al 2009 JMB) and this discrepancy is not addressed. At the very least the authors need to do a C1 alignment of their particles. There is a lot of focused reconstruction and symmetry averaging here, whereas a simple C1 would support evidence of 15 fibers. Validation studies (biochemically, or some type of labeling via cryo-EM) would be crucial to do. Further, 15 is a very strange symmetry mismatch, so this needs to be well supported with proper controls in order to state this claim. How the beta rich N-terminal domain can be elongated along the out ring shown in Supplementary Figure 8 and also form the tight coiled coil domains in Supplementary Figure 7 is not clear.
• Discussion: The authors state "The mechanisms of Schitoviridae attachment to bacteria are poorly understood. N4 binds the E. coli membrane protein NfrA 47 via its tail sheath 48, which surrounds the tail tube. However, this attachment mechanism is unlikely for DEV that lacks a tail sheath." They tested the LPS knockouts (Fig 4)…why not directly test an NfrA knockout too?
• Main Figure 4: needs better labels as non-phage readers will not be able to follow this. For example the wzy- data are not obvious, why there are only 2 panels of spot tests, yet three types of phage shown is not clear, the LPS molecule needs labels to show where the O-antigen is, and the dilutions should be labeled on the spot tests.
• Figure 6: panel A is not necessary. The left and middle gels are not needed at all. The right gel could be moved to supplemental. However, the real problem with this figure is that the data shown in panel B do not match the quality of the density shown in panel C. 2D class averages of an entirely helical structure should have obvious evidence of secondary structure even at modest to low resolutions of 9-10Å. These averages are a smear.
• Figure 8: There is no direct evidence shown that supports the location of gp71 (the vRNAP motor). Also given the uncertainty of the actual number of gp53 copies—see point above—the current model may be misleading.

MINOR ISSUES:
• Introduction: "The similarity of DEV to phage N4 is low"… can you provide a range or a value? What is considered low to some fields is different than others.
• Results: "DEV capsid is built by 535 copies of the major coat protein (T = 9) (Fig. 1C), with one penton replaced by the portal proteinm." The way this is written it seems a penton is replaced by a single monomer. Perhaps use "portal complex" instead? Or "12 copies of portal"? Protein is

also misspelled.
• Results: The authors state: “An initial dataset of gp72:gp73 collected on a 200 kV cryo-electron microscope gave convincing evidence that the two proteins form a tube-like structure, more extended than gp72 alone.” This isn’t really needed as these data are not shown. Only the 300 keV data are, so this sentence could be omitted.
• In the Results section all of the “correlation coefficients” are listed as 0.89 which does not match the data in Table 1. Check values.
• The paper has some weird phrasing and non standard English throughout. Example: “paves the ground” in the Discussion.
• Materials and Methods: “In replicate experiments, we found that between 7 and 24% of plaques were formed by phages unable to reproduce in the absence of pD53.” Please describe the statistics used. Otherwise it is hard to know if 7-24% is a reasonable number.

Reviewer #2 (Remarks to the Author):

This manuscript describes the high resolution structure determination of Pseudomonas phage DEV. This phage is a representative of the N4-like phage family (Schitoviridae). This family is quite distinct among podophages and has many interesting features that make it a worthy object of study. This is the first high resolution structure of an N4-like phage.

Overall, this study is well performed and presents a great deal of very interesting structural information. I believe that the data presented will be of interest to wide readership. My main criticisms of the manuscript, as outlined below, are with the general vagueness of the writing, especially in referring to previous work. I believe that the authors can greatly strengthen the manuscript with a more detailed description of previous knowledge and comparison of other systems with this one.

One other general point, the authors do not explain what conserved features define the Schitoviridae family. In particular, it would be interesting to know which structural features are conserved among all of these phages and which are most variable.

Detailed Comments:

Line 124: It is difficult to understand what the HF particles are. Since their heads must be expanded and tails are attached, it seems that they would have to have packaged DNA in their heads. Did the authors see 2 distinct bands in CsCl gradients? Wouldn’t this be expected with such a difference in DNA content? Did the authors run the DNA from these purified phages on a gel? Since these strange particles were observed, the authors should do a few simple experiments to provide some hypotheses for the origin of these particles. The reference to phage SU10 and similar cables is completely unhelpful. What is phage SU10? What were the cables?

Line 130: Were these particles proheads or mature phages that subsequently lost their DNA. The description of these particles only adds to the confusion. How could the cables be involved in assembly if they are made of DNA? Are the authors sure that the cables are made of DNA? Could it be scaffold protein? Presenting data about the HF particles and then not making more

of an effort to interpret what the particles are leaves the reader rather unsatisfied.

Lines 175-183: The discussion of the HT-adaptor would not be easy to appreciate for a general reader. It seems that the main point is that the DEV HT adaptor looks like the first ring of the head-tail joining complex that is seen in many podo, sipho, and myophages. The authors should clearly make this point. Simply naming two phages with the most similar HT-adaptors is not informative for most readers. This is something that the authors should generally correct in this manuscript. Saying that the structure of a DEV protein is similar to some other phage without any explanation or figure to show what is similar is not helpful. Comparisons should help the reader appreciate what might be general themes among diverse phages.

Lines 186-203: In discussing the "tail tube" of podophages the authors should point out that the tail tube of these types of phages are not at all like the tail tubes of sipho- and myophages. I think many readers will associate the term tail tube with these long-tailed phages. It would be useful for the authors to distinguish these different types of tail tubes and discuss whether tail tubes are a universal feature of podophages.

Line 203: The authors mention the "tail needle" of phage P22 without explaining what this is. For a general journal like Nat Comms, the authors should assume that readers are unfamiliar with these terms and phages. I am a phage biologist, but I do not know how the tail needle of P22 compares to the tail tube of DEV. These authors must elaborate on these points with figures and more explanation so that all readers can appreciate the importance of these comparisons.

Line 222: Again the "tail needle" is mentioned without an explanation or figure to compare.

Lines 258-259: "like in the podophage GP4" -This is another meaningless statement. Is there a 15:12 symmetry mismatch in this case? Again, the authors must explain what they mean, not expect the reader to be able to figure it out.

Line 279: The CRISPR-based mutation screening approach is nice, and others may want to use it. What was the frequency of mutants that were unable to reproduce without pD53? Stating this somewhere would give the reader an idea of how hard it was to find the mutant you wanted.

Line 284: "LOS" is not defined.

Lines 302-304: The comparison to the T7 injection protein is impossible to interpret. The level of protein similarity quoted is way below what anyone would consider significant, especially since the authors did not state the length of the alignment being used. Is there any other evidence (e.g. alphafold or HHpred) to support the idea that these proteins are similar in structure. Are the authors even trying to suggest that these proteins are similar in structure? Again, the vagueness of the comparison provides no information at all.

Line 314: "morphologically similar to gp15" -This is another completely vague statement. In exact terms, what is actually similar between these proteins? A figure would be useful. What is the meaning of this similarity?

Lipid Bilayer Experiments: I had trouble interpreting these experiments as I am not familiar with the technique. I expect many other readers will also be unfamiliar. The authors have no positive

control. What level of current would be expected in an assay like this if a proven pore forming protein were used? Since gp73 and gp72 likely will be in a complex during phage infection, what is the significance of gp73 forming a pore while the gp72/gp73 complex does not? “These experiments conclusively demonstrate that the gp72-gp73 complex forms a membrane-spanning structure capable of translocating DNA across the outer membrane of P. aeruginosa.” is a huge overstatement. I would say “suggest” is a more appropriate verb to use here.

Lines 418-420: The comparison with phage N4 is confusing. The use of the term “tail sheath”, even though published elsewhere, should be strongly qualified. This tail sheath is nothing like the tail sheath that is seen in all myophages, and the function is surely not the same either. There is no high resolution structure of phage N4 and what those authors called a “tail sheath” is probably some kind of tail fibre that happens to be packed closely to the tube. The authors should make a more precise discussion of this issue and emphasize that this use of the term “tail sheath” is not referring to a typical phage tail sheath. This mixing up of terminologies is not at all helpful to the field. There is no reason that the poor naming choice used in a previous publication needs to be propagated without comment or correction.

Lines 433-435: The authors seem to be saying that the second receptor is a protein, but couldn’t it be the inner part of the LPS? “that binds receptors in other podophages” – I don’t know what this means. The authors should explain this point.

Lines 452-454: Again, this conclusion is stated with too much certainty. The data presented is suggestive not convincing.

Lines 454-455: What podoviridae is being referred to? What is actually know about these proteins? Could their structures be similar?

Reviewer #3 (Remarks to the Author):

The manuscript “Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor” by Lokareddy et al. reports the structure of N4-like phage DEV, including proteins involved in channel formation for genome delivery. More intriguingly, the authors identify another function for the large virion-associated RNA polymerase encoded by these phages.

The structures are beautiful and the analysis is thorough. I have one major point about the lipid bilayer experiments, which I think would be helped by explaining the method in somewhat greater detail. As written, I am not confident in my ability to interpret some of the results presented in Figure 7C-E. Although the method was previously published, more information in the current manuscript would be greatly appreciated to help readers along.

I have other minor comments on the presentation.
1. Lines 120-128: Are the cables thicker in HF capsids than the DNA in FF capsids?

2. Figure 1C: The asymmetric subunit as shown is difficult to interpret. This may be a simple

issue like needing darker colors, or showing just the proteins outside of the map. Since the capsid protein is already shown to have good fit inside the density on the left, it may not be necessary to include the full density on the right.

3. Supplemental Figure 3: In the PDF version, some of the labels in the figure were given line numbers – not the fault of the authors, but it made the figure really confusing! My real comment is that in the legend, maybe replace “in this paper” with “in this work.”

4. Lines 433-435: This sentence is confusing as written. It may just have too many commas. Maybe something like “Thus, DEV can bind directly to the secondary receptor when accessible using another receptor-binding protein. This is likely the gp56 short tail fiber, which is known to bind receptors in other podoviruses.”

5. After reading the full paper, there may be a disconnect between the body of work and the abstract. As written, the abstract suggests the vRNAP is either the sole ejection protein, or that it is responsible for injecting the genome. I think this can be fixed in the statement “genome ejection motor across the host cell envelope” by clarifying that the vRNAP is part of the ejectosome, that it is embedded in the membrane but primarily cytoplasmic, and that it pulls the genome into the cell during transcription.

Point-by-point response to reviewer comments
We thank the reviewers and the editor for the thoughtful review of our paper. We have done our best to address all the reviewers' comments. Below, we provide a point-by-point response: all edits are marked in red in the revised manuscript. Major changes included in the revised paper are:

1 – We added a Mass Spec analysis of DEV structural proteins (**Table 1**) and SDS-PAGE of virions used for cryo-EM (**Supplementary Fig 1A**).

2 – We conducted a biochemical analysis of DEV 3,398 residues virion-associated RNA polymerase, gp71. We discovered that gp71-C associates with the periplasmic tunnel (PT) gp72 (**Fig. 8A-C**) that, in turn, form a complex with the outer membrane pore (OMP) gp73. Thus, we have deciphered the complete topology of DEV DNA-ejectosome, which we hypothesize is conserved in the *Schitoviridae* family.

3 – We performed a phylogenetic analysis of DEV's newly discovered ejection proteins gp71, gp2, and gp73 in *Schitoviridae*, revealing that these proteins are conserved yet diverging in sequence and structure. We included a new section '***An ancient origin for the Schitoviridae ejection apparatus***' on page 13 and new **Fig. 8D, Supplementary Fig.12, and Supplementary Table S5.**

Reviewer #1
The paper "Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor" by Lokareddy et al reports on a high-resolution structure for phage DEV virions using cryo-EM methods. In addition, protein purification of two ejection proteins and lipid bilayer experiments show that these proteins form pores starting from the outer membrane of the host. Phage plating experiments are done to probe the primary receptor for interactions with the host. Overall, most of the work is of high quality and this study is an important area of research as to date this is the first reported high-resolution structure for an N4-like phage, which is a type of podophage quite different than other well described podophages (e.g. P22, Sf6, T7). Largely the paper is well written and enjoyable to read.
Thank you!

However, there are some issues that detract from the work and some conclusions that are not well supported by the available data. These need to be addressed before the work is of suitable publication quality. I have broken down these points into major and minor issues and describe each below.
One overarching concern is uncertainty over the presence and/or copy number of several proteins. Specific examples are listed below. In general, thorough biochemical analyses such as gels and mass spectrometry would help strengthen several hypotheses generated by the cryo-EM density.
Agreed. *We included Mass Spec (**Table 1**) and SDS-PAGE (**Supplementary Fig. 1A**) analyses of DEV virions used for cryo-EM. A complete description of the new data (page 3, lines 104-110) and relative methods (page 19) is also provided.*

In addition, currently the paper is targeting an audience that is phage-savvy and structure savvy, and more general information needs to be provided to make the work accessible to the broad readership of Nature Communications. I think it would be wise to move a portion of the Discussion (e.g. the first paragraph) upfront to the Introduction so the significance and importance is more obvious right away.
Agreed. *We moved a portion of the Discussion to the Intro and expanded on the biology of N4 (page 2, lines 58-77).*

In addition, some figures can be removed to highlight the more important points of the paper and not overwhelm the reader with details. Likewise, the overabundance of supplementary data makes the reader flip between the main and supplemental section so often if becomes tedious.
Agreed. *We removed former Supplementary Figures: S1E, S4, S11, S7B and S9C*. See below for details.
I'd suggest the following figure removals:
1. Supplementary Figure 1: Other than panel A this figure does not add much. Also, this observation of partial and fully filled capsids takes on a substantial portion of the Results section and is the first thing presented, but this phenomenon is likely not physiologically relevant whatsoever.

Done. ***Figure S1E was removed.*** We kept panels A-D (now B-E) to demonstrate that the two DEV populations identified on the grid, HF and FF, are sufficiently homogeneous for single-particle analysis.

2. Supplementary Figure 4: redundant with Fig 1 in the main text. What does this add?

Done. ***We removed (former) Supplementary Figure 4.***

3. Not sure what Figure 3 adds, especially given the uncertainty of the stoichiometry.

Figure 3 points out a major difference between *Pseudomonas* phage DEV and *E. coli* N4, which has twelve appendages. We clarify this point by adding a new panel 3C and relative text on page 8, lines 288-309.

MAJOR ISSUES:

• Abstract: The authors state: "We identified DEV ejection proteins and, unexpectedly, found that the giant DEV RNA polymerase, the hallmark of the Schitoviridae family, is an ejection protein." This is not novel information as it has been well-established that vRNAPs are ejected into the hosts for both T7 and N4. As far as labeling it an "ejection protein" and part of the motor…. the data shown for this claim within this paper are very weak/nonexistent (see below).

We disagree. The reviewer is missing the point that we have strengthened in the revised paper. We are obviously aware that the N4 vRNAP was shown to be ejected into the host, as referenced in the paper several times [1] [2]. However, not all proteins ejected from a virion upon infection are also "Ejection Proteins". For instance, phage P22 ejects the tail needle gp26 into *Salmonella* upon infection, but the tail needle is ***not*** an Ejection Protein. As of 2024, the definition of Ejection Protein is functional: *a set of virion-associated proteins ejected upon infection to form a transmembrane channel in the host cell envelope, which mediates genome ejection* [3]. My lab has characterized phage T7 [4] [5] and P22 [6] Ejection Proteins at the biochemical and structural level, and in this paper, we discover N4 homologous proteins. We demonstrate that DEV vRNAP (gp71) is part of an operon encoding the previously unidentified Ejection Proteins gp72 and gp73. We significantly expanded this finding in the revised paper, where we show that gp71-C physically associates with gp72 (**Fig. 8A-C**) and DEV operon encoding ejection proteins is evolutionarily conserved in >190 genomes of *Schitoviridae* (see page 13 and **Fig. 8D**). Thus, this paper provides a roadmap to decipher Ejection Proteins biology in *Schitoviridae*. ***We reworded the Abstract to emphasize this concept.***

• Results: The authors state "Unfortunately, this density did not align with the capsid or portal axis and could not be improved. It likely belongs to the 3,398 amino acid vRNAP, as proposed for the phage N4 6." There is really no evidence for this claim. What is the calculated volume of this density? Does it correspond to the known size/copy number of the polymerase? What is the calculated volume of this density? Does it correspond to the known size/copy number of the polymerase? While this could be the location, the authors have done a lot with symmetry and focused reconstruction methods that this could be entirely artifactual. A knockout would need to be studied and C1 reconstructions used for accurate comparisons.

Agreed. We have removed (former) Supplementary Figure 4 and the sentence criticized by the reviewer. Unfortunately, we cannot knock out DEV vRNAP/gp71 at this time. We are working on a cryo-EM reconstruction of the isolated vRNAP, which will be presented elsewhere.

• Results: The authors state "This suggests the cables do not play a structural role or stabilize the capsid from the inside but perhaps are involved in capsid assembly." This is a wildly inaccurate statement. Most if not all dsDNA phages first assemble to form procapsid shells and then the genome is packaged via a packaging terminase motor. How could the genome "cables" possibly be involved in assembly?

We disagree with the reviewer who is extrapolating Schitoviridae biology based on no data. Truthfully, absolutely nothing is known about the assembly of *Schitoviridae* capsids. A scaffolding protein has not been identified in the N4-literature. Our initial hypothesis (which I presented at the 2023 Phage and Virus Assembly meeting in the UK) was that DEV cables represent a scaffolding protein (or a fragment of it) that persists in the mature virion. We scrutinized all ORFs near the coat and portal to test this hypothesis and generated AlphaFold models. We found two ORFs between DEV gp77 (coat) and gp80 (portal), namely, gp79, encoding a 112 amino acid fully helical protein, and gp78, encoding a 396 amino acid also enriched in α-helices and coiled regions. However, MS did not identify either protein in the mature virion (e.g., this information is now in **Table 1**). Thus, we believe the cable is just DEV DNA left inside the capsid. We made the following changes to the revised paper: ***(i) We removed the sentence***: "*This suggests the cables do not play a structural role or stabilize the capsid from the inside but perhaps are involved in capsid assembly*".

(*ii*) ***We expanded the analogy*** between DEV and SUP10 cables: see page 4, lines 147-152.
(*iii*) ***We added new text on page 5***, lines 166-171, to clarify that the cables are likely DNA left in the capsid.
(*iv*) ***We provide a new Supplementary Fig. 4C*** that clarifies the orientation of DEV cables inside the capsid.

• Results: The authors state "The lack of physical constriction and the continuous DNA density inside the tail tube suggests that the DEV tail is sealed by a plug at the distal tip relative to the capsid, analogous to the tail needle of phage P22 21 and Sf6 20." The data shown in Supplementary data Figure 4B are not convincing that there is strong density here. Also, biochemical analysis would show if there was a protein positively identified in the mature virion that is not accounted for by the other assigned cryo-EM density.

We agree with the reviewer that the density for the tail fiber gp56 is not well resolved, likely due to its intrinsic dynamicity and our inability to align it during single particle analysis. ***However, the tail tube channel is plugged:*** see **Fig. 2C**, ***Supplementary Fig. 5B, Supplementary 6C.*** Additionally, the idea that gp56 forms the plug is bolstered by the fact that we identified gp56 by MS analysis as one of the DEV structural proteins (**Table 1**). Accordingly, gp56 orthologue was identified among LIT1 virion proteins [7]. ***This information is now in Table 1.*** We also identified gp56 as a faint band by Coomassie staining, suggesting it is part of the virion (**Supplementary Figure 1A**). ***We clarified these points on page 6, lines 231-235 and 237-239.***

Additionally, we knocked out gp56 and found that DEV gp56-minus particles are largely devoid of DNA, consistent with the expected 'DNA-leakage phenotype' (**Fig. R1**). However, gp56-minus phages (Δgp56) can only be complemented by the entire gp54-gp55-gp56 operon. Notably, gp54 (223 residues), but not gp55 (99 residues), is found in the MS analysis of DEV mature virion (**Table 1**). An AlphaFold model of gp54 reveals a similar fold to the sheath protein of phage N4, which we visualized at 3 Å resolution in my lab using cryo-EM. Our working model is that gp54 binds the plug gp56 and functions as the secondary receptor-binding protein. These data were just submitted for publication and will not be included in the revised paper. Thus, as hypothesized in this paper, we are confident that gp56 functions as DEV's plug and is (indirectly) involved in binding to a secondary receptor.

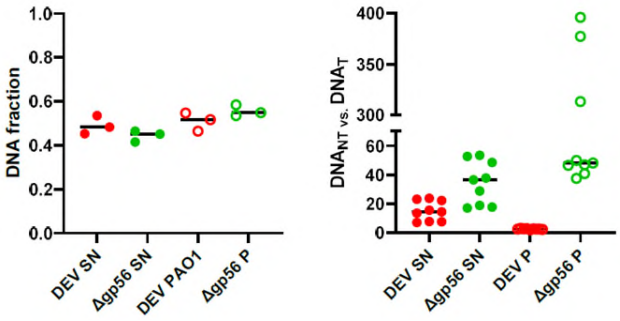


Fig. R1. Phage particles in lysates of DEV (red symbols) or DEV Δgp56 (green symbols) were pelleted by PEG - NaCl precipitation. Both the supernatants and the pellets were analyzed by Real-Time PCR with a pair of phage-specific oligonucleotides before and after treating the samples with DNase I, which degrades only un-packed DNA or DNA released from particles, but not DNA inside the phage head. Left panel: relative amount of phage DNA in pellets (P) and supernatants (SN) before the treatment with DNase I. Right panel: Relative amount of phage DNA content in samples not treated with DNase I vs. treated samples. The proportion of DNA found in both the pellet and supernatant is comparable for both phages, indicating that the absence of gp56 does not affect DNA packaging. However, DNase I treatment significantly reduced the DNA content in both supernatants, while its impact on the DEV pellet was much lower (an average decrease of 2.6±0.29 fold between treated and untreated samples). Conversely, DNase I degraded the DEV Δgp56 DNA present in the pellet, indicating that the phage genome had (partially) leaked from the phage particles.

• Results: I have trouble with the entire sub-section entitled "DEV long-tail fiber gp53 assembles to the tail via a 15:12 symmetry mismatch". This is very different than seen for N4 (Choi et al 2009 JMB) and this discrepancy is not addressed. At the very least the authors need to do a C1 alignment of their particles. There is a lot of focused reconstruction and symmetry averaging here, whereas a simple C1 would support evidence of 15 fibers. Validation studies (biochemically, or some type of labeling via cryo-EM) would be crucial to do.

Agreed. We obviously tried to compute a C1 map, but the appendages are too weak to align. The C1 map only shows a strong 'collar' density around the tail tube (shown in **Supplementary Fig 5B**). Labeling the appendages is pointless if the signal is smeared (shown in **Supplementary Fig 6A,B**). We used functional validation to fill the gaps the inconclusive structural characterization left. We knocked out gp53 (**Fig. 4**) and tested the gp53-minus mutant functionally, which led us to discover that gp53 is DEV's primary LPS binding receptor. ***We clarified this point in the revised text on page 7, lines 242-243; 247-248, and 251. We also added a new Supplementary Fig. 5B that illustrates the C1 reconstruction requested by the reviewer.***

In addition, the reviewer keeps referring to N4 as if this E. coli phage was a replica of DEV. This is genuinely inaccurate. We have just solved a 3 Å structure of N4 that is drastically different than DEV. N4 has 12 very visible appendages (easy to align in a C1 reconstruction), an unusual plug sealing the tail tube, and two sheath proteins surrounding the tail tube.

Further, 15 is a very strange symmetry mismatch, so this needs to be well supported with proper controls in order to state this claim. How the beta rich N-terminal domain can be elongated along the out ring shown in Supplementary Figure 8 and also form the tight coiled coil domains in Supplementary Figure 7 is not clear.
Agreed. DEV gp53 has a flexible linker of 22 residues (res. 92-114) connecting the N-terminal β-barrel (res. 1-91) to the trimeric coiled-coil (res. 115-250). We believe this linker allows for the mismatch between NTBs and trimeric coiled coils to occur. A similar 15:12:5 symmetry mismatch was found in the podophage GP4 [8]. ***We clarified these points in the revised paper on page 8, lines 288-309, and we added a new Fig. 3C to clarify the proposed symmetry mismatch.***

• Discussion: The authors state "The mechanisms of Schitoviridae attachment to bacteria are poorly understood. N4 binds the E. coli membrane protein NfrA 47 via its tail sheath 48, which surrounds the tail tube. However, this attachment mechanism is unlikely for DEV that lacks a tail sheath." They tested the LPS knockouts (Fig 4)…why not directly test an NfrA knockout too?
Unfortunately, this experiment is impossible. *P. aeruginosa* lacks a *nfrA (and nfrB)* orthologue. ***We clarify this point on page 15, lines 572-574.***

• Main Figure 4: needs better labels as non-phage readers will not be able to follow this. For example, the wzy-data are not obvious, why there are only 2 panels of spot tests, yet three types of phage shown is not clear, the LPS molecule needs labels to show where the O-antigen is, and the dilutions should be labeled on the spot tests.
Done! We revised panel 4C and the figure caption.

• Figure 6: panel A is not necessary. The left and middle gels are not needed at all. The right gel could be moved to supplemental. However, the real problem with this figure is that the data shown in panel B do not match the quality of the density shown in panel C. 2D class averages of an entirely helical structure should have obvious evidence of secondary structure even at modest to low resolutions of 9-10Å. These averages are a smear.
Done! We moved panel A to ***Supplementary Fig. 9A.*** Regarding the 2D classes in Fig. 6D, they are not a smear. Perhaps the low resolution of the PDF file reduces the actual quality of this image. A smear would not yield a 3.1 Å reconstruction (9-fold symmetrized). We provide an improved set of 2D class averages (now **Fig. 6A**). ***We also released the electron density map EMD-43629 and relative PDB models of the gp72:gp73 complex. The density is good except for gp53 N-terminal 52 amino acids that are disordered. We state this point in the revised text on page 11, line 413.*** Structural studies in lipid nanodiscs are ongoing in my lab.

• Figure 8: There is no direct evidence shown that supports the location of gp71 (the vRNAP motor). Also given the uncertainty of the actual number of gp53 copies—see point above—the current model may be misleading.
Agreed. We vastly expand the characterization of DEV gp71 and have more substantial evidence supporting the model in **Figure 9**. ***First***, the association of N4 vRNAP with the cytoplasmic bacterial membrane has been documented [2]. Both N4 gp50 and DEV gp71 contain a predicted transmembrane region in the first 800 amino acids and lack cysteines. We describe this biochemical property on page 12, lines 471-476. ***Second***, we conducted biochemcial mapping of vRNAP (gp71) domains and found the C-terminal domain assembles with gp72 (aww new **Fig. 8A-C**). These new data are described in a new section, '***Domain analysis of DEV ejection protein gp71***' on page 12, line 462. Thus, we have now mapped the complete topology of the DEV DNA ejectosome, as shown in **Figure 9**. ***Third***, we also carried out an evolutionary analysis of *Schitoviridae* ejection proteins and found they are conserved in >190 genomes (page 13, section '***An ancient origin for the Schitoviridae ejection apparatus***'). We found that the lack of cysteine residues is a conserved feature of gp71-homologs (page 14, lines 542-556), reflecting the requirement for passage through the host periplasm.
However, we do agree with the reviewer that **Figure 9** is just a model, as stated repetitively in the text. Thus, we revised the Figure 9 caption by stating, "***Proposed model for DEV absorption onto P. aeruginosa surface and genome ejection*".**

MINOR ISSUES:

• Introduction: “The similarity of DEV to phage N4 is low”… can you provide a range or a value? What is considered low to some fields is different than others.

Done. See lines 80-83 on page 2-3.

• Results: “DEV capsid is built by 535 copies of the major coat protein (T = 9) (Fig. 1C), with one penton replaced by the portal protein.” The way this is written it seems a penton is replaced by a single monomer. Perhaps use “portal complex” instead? Or “12 copies of portal”? Protein is also misspelled.

Fixed. Thank you!

• Results: The authors state: “An initial dataset of gp72:gp73 collected on a 200 kV cryo-electron microscope gave convincing evidence that the two proteins form a tube-like structure, more extended than gp72 alone.” This isn’t really needed as these data are not shown. Only the 300 keV data are, so this sentence could be omitted.

Removed, thank you!

• In the Results section all of the “correlation coefficients” are listed as 0.89 which does not match the data in Table 1. Check values.

Fixed, thank you.

• The paper has some weird phrasing and nonstandard English throughout. Example: “paves the ground” in the Discussion.

Fixed. Also, the revised paper has been thoroughly proofread.

• Materials and Methods: “In replicate experiments, we found that between 7 and 24% of plaques were formed by phages unable to reproduce in the absence of pD53.” Please describe the statistics used. Otherwise, it is hard to know if 7-24% is a reasonable number.

Done. See page 20, lines 767-773.

Reviewer #2

Overall, this study is well performed and presents a great deal of very interesting structural information. I believe that the data presented will be of interest to wide readership. My main criticisms of the manuscript, as outlined below, are with the general vagueness of the writing, especially in referring to previous work. I believe that the authors can greatly strengthen the manuscript with a more detailed description of previous knowledge and comparison of other systems with this one.

Thank you! In the revised manuscript, we thoroughly explain and clarify all points the reviewer felt were vague.

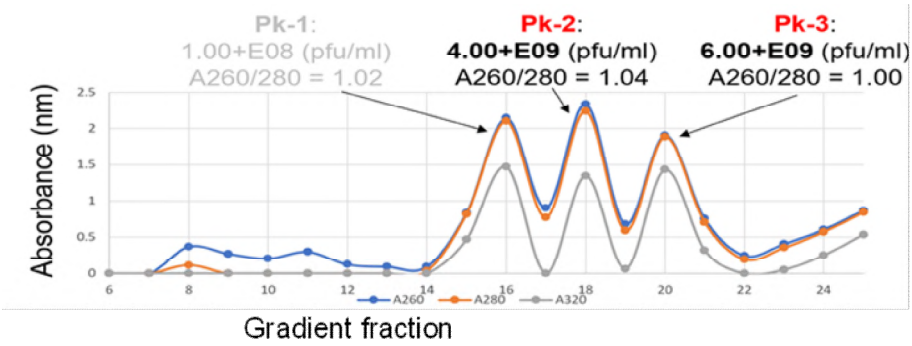
One other general point, the authors do not explain what conserved features define the Schitoviridae family. In particular, it would be interesting to know which structural features are conserved among all of these phages and which are most variable.

Agreed. We performed an extensive bioinformatics and evolutionary analysis of ejection protein conservation in the *Schitoviridae* family. We added a new section on page 13, line 489: “***An ancient origin for the Schitoviridae ejection apparatus****”, a new* ***Figure 8D,*** *and two new* ***Supplementary Figures 10 and 12****. The new data strongly support the hypothesis that ejection proteins are functionally and structurally conserved in the Schitoviridae family. We also emphasize this point in the Abstract (page 1, lines 36-40) and Discussion (page 16, lines 605-621).*

Detailed Comments:

Line 124: It is difficult to understand what the HF particles are. Since their heads must be expanded and tails are attached, it seems that they would have to have packaged DNA in their heads. Did the authors see 2 distinct bands in CsCl gradients? Wouldn’t this be expected with such a difference in DNA content?

Agreed. Both HF and FF are expanded. We did not see two distinct bands in the $CsCl_2$ gradient, whereas we saw different peaks by purifying phages on sucrose gradients (shown below). Pk2 was enriched in HF particles, whereas Pk3 contained mainly FF virions.



Did the authors run the DNA from these purified phages on a gel? Since these strange particles were observed, the authors should do a few simple experiments to provide some hypotheses for the origin of these particles.

Agreed. We tried all sorts of experiments to identify the molecular nature of DEV cables. The featureless cable density, which looks like a sausage, can fit either double-stranded DNA or a α-helical protein. As described above in response to reviewer #1, we failed to identify a putative scaffolding protein in DEV that could explain the molecular identity of the cables inside the capsid. Nonetheless, the cables are prominent in DEV HF and FF capsids. Failure to find a protein that accounts for the cable density led us to hypothesize that the cable is double-stranded DNA left in the capsid. Accordingly, HF particles have an A260/A280 = 1.04, suggesting the presence of some nucleic acids. However, when analyzed by agarose gel electrophoresis, HF particles did not show a low M.W. DNA/RNA band, suggesting the nucleic acids putatively present inside the capsid have different sizes and perhaps are too small to be visualized. ***Thus, we are not sure about the chemical identity of DEV cables***. However, *we do not want to omit this information from the revised paper, as the cables are part of DEV HF particles and exist in FF virions, though buried by bulk DNA*. We found a similar cable density in the N4 virion, which we have visualized at atomic resolution using cryo-EM (Bellis *et al.*, *in preparation*). Cables were also observed in the E. coli phage SU10 reconstruction, but no molecular identity was assigned to these structures. We hope our work will prompt other researchers to observe and identify the molecular nature of cables. Science is incremental by nature, and others will clarify what we observe and report in this study. ***We clarify the putative nature of DEV cables on page 5, lines 166-171.***

The reference to phage SU10 and similar cables is completely unhelpful.

We disagree. Omitting to cite a paper that has made a similar observation and was published in the same journal, Nature Communications, just 1.5 years ago [9], would be a disservice to the scientific community, even if the authors of this work did not explain the chemical identity of SU10 cables.

What is phage SU10? What were the cables?

Phage SU10 is an *E. coli* phage of the Kuravirus genus. It is characterized by a short non-contractile tail (e.g., *Podoviridae*), a prolate head, and a large genome of 77,327 base pairs [10]. A recent cryo-EM reconstruction of the virion before and after genome ejection revealed characteristic cable-like structures inside the prolate capsid, concentric to the portal protein, thought to be remnants of the genome left in the capsid after genome ejection [9]. ***We added this information to the revised paper on page 4, lines 147-152.***

Line 130: Were these particles proheads or mature phages that subsequently lost their DNA. The description of these particles only adds to the confusion.

In the revised paper, we state (***page 3, lines 116-117***) that both HF and FF are mature capsids.

How could the cables be involved in assembly if they are made of DNA? Are the authors sure that the cables are made of DNA? Could it be scaffold protein? Presenting data about the HF particles and then not making more of an effort to interpret what the particles are leaves the reader rather unsatisfied.

The new text on ***page 4, lines 147-152, and page 5, lines 166-171,*** should answer the reviewer's questions/concerns.

Lines 175-183: The Discussion of the HT-adaptor would not be easy to appreciate for a general reader. It seems that the main point is that the DEV HT adaptor looks like the first ring of the head-tail joining complex that is seen in many podo, sipho, and myophages. The authors should clearly make this point. Simply naming two phages with the most similar HT-adaptors is not informative for most readers. This is something that the authors should generally correct in this manuscript. Saying that the structure of a DEV protein is similar to some other phage without any explanation or figure to show what is similar is not helpful. Comparisons should help the reader appreciate what might be general themes among diverse phages.
Agreed. We have sharpened the description of the DEV HT-adaptor (***page 6, lines 204-212***) and provided a better visual representation of the DEV HT-adaptor fold in ***Supplementary Figure 3***.

Lines 186-203: In discussing the "tail tube" of podophages the authors should point out that the tail tube of these types of phages are not at all like the tail tubes of sipho- and myophages. I think many readers will associate the term tail tube with these long-tailed phages. It would be useful for the authors to distinguish these different types of tail tubes and discuss whether tail tubes are a universal feature of podophages.
Agreed. Excellent point. See lines 216-219 on page 6!

Line 203: The authors mention the "tail needle" of phage P22 without explaining what this is. For a general journal like Nat Comms, the authors should assume that readers are unfamiliar with these terms and phages. I am a phage biologist, but I do not know how the tail needle of P22 compares to the tail tube of DEV. These authors must elaborate on these points with figures and more explanation so that all readers can appreciate the importance of these comparisons.
Line 222: Again the "tail needle" is mentioned without an explanation or figure to compare.
Agreed. The phage tail needle has been widely documented in the literature, described in dozens of papers, and cited hundreds of times, the same as the sheath protein or a tailspike. *Nonetheless, to better clarify what the tail needle is/does, we added the following statement (**page 6, lines 237-239**):* "The lack of physical constriction and the continuous DNA density inside the tail tube suggest that the DEV tail is sealed by a plug at the distal tip relative to the capsid. ***We hypothesize that this plug could be analogous to the tail needle of podophages P22 [6] and Sf6 [11], which seals the tail after genome packaging, stabilizing encapsidated DNA [12]".***

Lines 258-259: "like in the podophage GP4" -This is another meaningless statement. Is there a 15:12 symmetry mismatch in this case? Again, the authors must explain what they mean, not expect the reader to be able to figure it out.
Agreed. We greatly expanded on this point in the revised paper and thoroughly clarified the strong analogy between DEV and GP4, which was described in a paper published in 2023 in *J Mol Biol.* Please see on ***page 8, lines 288-309***. We also added a new schematic of gp53 attachment to the HT-adaptor (**Figure 3C**) that exemplifies the proposed *12:15:pseudo-5* symmetry mismatch in the DEV tail.

Line 279: The CRISPR-based mutation screening approach is nice, and others may want to use it. What was the frequency of mutants that were unable to reproduce without pD53? Stating this somewhere would give the reader an idea of how hard it was to find the mutant you wanted.
Thank you! We added these data to the revised paper: see page 20, lines 767-773.

Line 284: "LOS" is not defined.
Fixed, thank you (page 3, line 89).

Lines 302-304: The comparison to the T7 injection protein is impossible to interpret. The level of protein similarity quoted is way below what anyone would consider significant, especially since the authors did not state the length of the alignment being used. Is there any other evidence (e.g. alphafold or HHpred) to support the idea that these proteins are similar in structure. Are the authors even trying to suggest that these proteins are similar in structure? Again, the vagueness of the comparison provides no information at all.
Line 314: "morphologically similar to gp15" -This is another completely vague statement. In exact terms, what is actually similar between these proteins? A figure would be useful. What is the meaning of this similarity?

Agreed. We added a comparative analysis of T7 gp15 and DEV gp72 in ***Supplementary Fig. 10*** and compared the two structures ***on pages 9-10, lines 360-369.*** *We also carried out an evolutionary analysis of DEV ejection protein conservation in >190 genomes of Schitoviridae. See the new section '***An ancient origin for the *Schitoviridae* ejection apparatus***'* *on page 13, Figure 8D and Supplemental Figure 12*.

Lipid Bilayer Experiments: I had trouble interpreting these experiments as I am not familiar with the technique. I expect many other readers will also be unfamiliar. The authors have no positive control. What level of current would be expected in an assay like this if a proven pore forming protein were used? Since gp73 and gp72 likely will be in a complex during phage infection, what is the significance of gp73 forming a pore while the gp72/gp73 complex does not? "These experiments conclusively demonstrate that the gp72-gp73 complex forms a membrane-spanning structure capable of translocating DNA across the outer membrane of P. aeruginosa." is a huge overstatement. I would say "suggest" is a more appropriate verb to use here.
Done! We have expanded the entire section describing the lipid bilayer assay on pages 11-12, lines 429-460. We performed our experiments with all necessary controls, i.e., we recorded current traces using a buffer control to exclude detergent interference. A positive control in lipid bilayer experiments would be another protein with known pore-forming activity and would only confirm that our system is capable of detecting pore-forming proteins. However, we know that our system reliably and reproducibly detects pore-forming proteins, as shown in this study and many other publications. Hence, positive controls are generally not published in lipid bilayer experiments. In principle, we could show these experiments with our standard pore-forming protein MspA, but these experiments would have no relevance for the Dev proteins analyzed in this study.

However, it is important to note that membranes in lipid bilayer experiments can vary from one experiment to another. This is the reason why we record multiple membranes for each sample and why we recorded. If a protein of interest is inserted into a lipid membrane and forms a channel, then one can observe an increase in current compared to the baseline when no protein is added. These stepwise increases in current or conductance are indicative of channel formation in the membrane and constitute a positive "control" *per se* provided that these events are reproducible with different membranes with different sample preparations, as was done in our study. Channel-forming proteins have a wide range of current amplitudes or conductances. For example, the Tsx channel of *E. coli* has conductance of 10 pS [13], the major porin of *E. coli* OmpF has conductance of 1500 pS [14], while the sugar transporter LamB of *E. coli* has conductance of 155 pS [15].

In our experiments, we observed a conductance of 300 pS (30 pA at 100 mV) for gp73, which is well within the range of observed conductances of other channel-forming proteins. The fact that we did not observe channel forming of gp72:gp73 complex in our lipid bilayer experiments might be caused by a structural change of gp73 in complex with gp72, which closes the channel to prevent loss of DNA but also precludes detection of channel activity. DNA ejection by the phage after binding to the cell surface of *P. aeruginosa* would open the channel, but this signal is missing in our lipid bilayer system. We rephrased this paragraph as suggested (lines 429-460).

Lines 418-420: The comparison with phage N4 is confusing. The use of the term "tail sheath", even though published elsewhere, should be strongly qualified. This tail sheath is nothing like the tail sheath that is seen in all myophages, and the function is surely not the same either. There is no high resolution structure of phage N4 and what those authors called a "tail sheath" is probably some kind of tail fibre that happens to be packed closely to the tube. The authors should make a more precise discussion of this issue and emphasize that this use of the term "tail sheath" is not referring to a typical phage tail sheath. This mixing up of terminologies is not at all helpful to the field. There is no reason that the poor naming choice used in a previous publication needs to be propagated without comment or correction.
We agree with the reviewer *but do not believe this paper is the right venue to rename N4 sheath protein.* Whether accurate or inaccurate, the definition of tail sheath is rooted in the N4 literature [16-19]. We have just determined a 3 Å cryo-EM structure of phage N4 and know the chemical identity of the N4 sheath precisely. It is formed by two subunits, not just one, as guessed based on a previous low-resolution analysis [17], and, as the reviewer suggested, it does not look like a Myoviridae/Syphoviridae sheath. We will keep the current nomenclature in the revised paper and propose a more rational name for this protein in the paper that describes the N4 structure. We revised the paper as follows (page 15, line 571): " ***N4 binds the E. coli membrane protein NfrA [20] via gp65 (e.g., tail sheath) [16], which surrounds the tail tube***. ***However, this attachment mechanism is unlikely for DEV, which lacks a homologous factor surrounding the tail tube***".

Lines 433-435: The authors seem to be saying that the second receptor is a protein, but couldn't it be the inner part of the LPS? "that binds receptors in other podophages" – I don't know what this means. The authors should explain this point.
Agreed. Thank you for this comment. We explicitly mention that a short LPS form may serve as a second receptor and rephrased the text (see **page 15, lines 581-585 and 590-593**).

Lines 452-454: Again, this conclusion is stated with too much certainty. The data presented is suggestive not convincing.
Agreed, we toned it down.

Lines 454-455: What podoviridae is being referred to?
Agreed. The comparison is with the Podophage T7. The text has been revised**.**
What is actually know about these proteins? Could their structures be similar?
We now cite a review on ejection proteins that we recently wrote [3]. We believe that all ejection proteins form a trans-envelope channel despite the minimal similarity in primary, tertiary, and quaternary structures (***see Supplementary Figure 10***). We hypothesize that convergent evolution may lead to reinventing a channel for DNA passage in the host cell envelope. We clarify these concepts on **pages 15-16, lines 612-621**.

Reviewer #3
The manuscript "Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor" by Lokareddy et al. reports the structure of N4-like phage DEV, including proteins involved in channel formation for genome delivery. More intriguingly, the authors identify another function for the large virion-associated RNA polymerase encoded by these phages.
Thank you!

The structures are beautiful, and the analysis is thorough. I have one major point about the lipid bilayer experiments, which I think would be helped by explaining the method in somewhat greater detail. As written, I am not confident in my ability to interpret some of the results presented in Figure 7C-E. Although the method was previously published, more information in the current manuscript would be greatly appreciated to help readers along.
Agreed. Also, in response to the reviewer's #2 criticism, we have expanded the description of the lipid bilayer method to clarify this methodology better. Please see lines 429-460.

I have other minor comments on the presentation.
1. Lines 120-128: Are the cables thicker in HF capsids than the DNA in FF capsids?
Yes, the cables are alike in HF and FF capsids.

2. Figure 1C: The asymmetric subunit as shown is difficult to interpret. This may be a simple issue like needing darker colors, or showing just the proteins outside of the map. Since the capsid protein is already shown to have good fit inside the density on the left, it may not be necessary to include the full density on the right.
Fixed. We removed the cryo-EM density, as the reviewer suggested. Thank you!

3. Supplemental Figure 3: In the PDF version, some of the labels in the figure were given line numbers – not the fault of the authors, but it made the figure really confusing! My real comment is that in the legend, maybe replace "in this paper" with "in this work."
Fixed. Thank you!

4. Lines 433-435: This sentence is confusing as written. It may just have too many commas. Maybe something like "Thus, DEV can bind directly to the secondary receptor when accessible using another receptor-binding protein. This is likely the gp56 short tail fiber, which is known to bind receptors in other

podoviruses."
Fixed. Rephrased as suggested (page 15, lines 581-584). Thank you!

5. After reading the full paper, there may be a disconnect between the body of work and the Abstract. As written, the Abstract suggests the vRNAP is either the sole ejection protein, or that it is responsible for injecting the genome. I think this can be fixed in the statement "genome ejection motor across the host cell envelope" by clarifying that the vRNAP is part of the ejectosome, that it is embedded in the membrane but primarily cytoplasmic, and that it pulls the genome into the cell during transcription.
Agreed. We reworded the Abstract to clarify that the vRNAP is part of the DEV DNA-ejectosome assembled across *P. aeruginosa* cell envelope. We also carried out an evolutionary analysis of Schitoviridae ejection proteins and found they are conserved in >190 genomes (page 13, section '***An ancient origin for the Schitoviridae ejection apparatus***'). We also performed a biochemcial mapping of vRNAP (gp71) domains and found the C-terminus of this protein assembles with gp72 (**Fig. 8D**). Thus, we have now mapped the complete topology of the DEV DNA ejectosome.

References

1. Falco, S.C., Laan, K.V. & Rothman-Denes, L.B. Virion-associated RNA polymerase required for bacteriophage N4 development. *Proc Natl Acad Sci U S A* **74**, 520-523 (1977).
2. Falco, S.C. & Rothman-Denes, L.B. Bacteriophage N4-induced transcribing activities in Escherichia coli. II. Association of the N4 transcriptional apparatus with the cytoplasmic membrane. *Virology* **95**, 466-475 (1979).
3. Swanson, N.A., Hou, C.D. & Cingolani, G. Viral Ejection Proteins: Mosaically Conserved, Conformational Gymnasts. *Microorganisms* **10** (2022).
4. Swanson, N.A. *et al.* Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 A resolution. *Mol Cell* **81**, 3145-3159 e3147 (2021).
5. Swanson, N.A. *et al.* Expression and purification of phage T7 ejection proteins for cryo-EM analysis. *STAR Protoc* **2**, 100960 (2021).
6. Iglesias, S.M. *et al.* Molecular architecture of Salmonella typhimurium virus P22 genome ejection machinery. *J Mol Biol*, 168365 (2023).
7. Ceyssens, P.J. *et al.* Molecular and physiological analysis of three Pseudomonas aeruginosa phages belonging to the "N4-like viruses". *Virology* **405**, 26-30 (2010).
8. Zheng, J. *et al.* Asymmetric Structure of Podophage GP4 Reveals a Novel Architecture of Three Types of Tail Fibers. *J Mol Biol* **435**, 168258 (2023).
9. Siborova, M. *et al.* Tail proteins of phage SU10 reorganize into the nozzle for genome delivery. *Nat Commun* **13**, 5622 (2022).
10. Khan Mirzaei, M., Eriksson, H., Kasuga, K., Haggard-Ljungquist, E. & Nilsson, A.S. Genomic, proteomic, morphological, and phylogenetic analyses of vB_EcoP_SU10, a podoviridae phage with C3 morphology. *PLoS One* **9**, e116294 (2014).
11. Li, F. *et al.* High-resolution cryo-EM structure of the Shigella virus Sf6 genome delivery tail machine. *Sci Adv* **8**, eadc9641 (2022).
12. Strauss, H. & King, J. Steps in the stabilization of newly packaged DNA during phage P22 morphogenesis. *J Mol Biol* **172**, 523-543 (1984).
13. Benz, R., Schmid, A., Maier, C. & Bremer, E. Characterization of the nucleoside-binding site inside the Tsx channel of Escherichia coli outer membrane. Reconstitution experiments with lipid bilayer membranes. *Eur J Biochem* **176**, 699-705 (1988).
14. Benz, R., Janko, K., Boos, W. & Lauger, P. Formation of large, ion-permeable membrane channels by the matrix protein (porin) of Escherichia coli. *Biochim Biophys Acta* **511**, 305-319 (1978).
15. Benz, R., Schmid, A., Nakae, T. & Vos-Scheperkeuter, G.H. Pore formation by LamB of Escherichia coli in lipid bilayer membranes. *J Bacteriol* **165**, 978-986 (1986).
16. McPartland, J. & Rothman-Denes, L.B. The tail sheath of bacteriophage N4 interacts with the Escherichia coli receptor. *J Bacteriol* **191**, 525-532 (2009).
17. Choi, K.H. *et al.* Insight into DNA and protein transport in double-stranded DNA viruses: the structure of bacteriophage N4. *J Mol Biol* **378**, 726-736 (2008).
18. Lenneman, B.R. & Rothman-Denes, L.B. Structural and biochemical investigation of bacteriophage N4-encoded RNA polymerases. *Biomolecules* **5**, 647-667 (2015).
19. Zhang, Z. *et al.* Characterization of Tail Sheath Protein of N4-Like Phage phiAxp-3. *Front Microbiol* **9**, 450 (2018).
20. Kiino, D.R., Licudine, R., Wilt, K., Yang, D.H. & Rothman-Denes, L.B. A cytoplasmic protein, NfrC, is required for bacteriophage N4 adsorption. *J Bacteriol* **175**, 7074-7080 (1993).

Reviewer #1 (Remarks to the Author):

Review for NCOMMS-24-08618 Revision

Generally the authors of "Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor" have responded well to the previous critiques and the revised paper is much better.

The C-terminal vRNAP binding data was an important addition to the paper, although I still find the author's conclusion that it is part of the "ejectosome" a bit of a stretch. The other proteins (gp72 and gp73) make a clear tube structure through the periplasm and resemble ejectosomes of other phages. I appreciate that the authors did a pull-down assay to show the C-terminus of gp71 interacts as the density in figure 6 is very weak and this bit of direct evidence strengthens their claims. However, regardless of it is in the same operon, and even though the gp71 protein likely associates as shown in the model in figure 9, classifying it as part of the ejectosome itself seems strange. It is ejected into the cytoplasm, but it is not spanning the periplasm like gp72/73.

MAJOR ISSUES:

• Figure 3 is hard to interpret as a static image. I requested the map and the fit of the Alpha Fold 2 model shown in 3A and this clarified a lot of points for me. So, I would suggest the authors make a movie for the supplemental data that shows a 3D rotation to accompany the figure to reduce confusion for readers. In addition, the C15 symmetrized map should be made publicly available in the EMDB and the statistics should be added to Table 2. This is important to make available and to report the stats since major conclusions are drawn from it. In my opinion, the static figure does an injustice to the structure (not the authors' fault—it is a hard angle to show in a single figure). They would showcase their work better as a movie.

MINOR ISSUES:
Line 75: No scaffolding protein was present in the bioinformatics, but were there genes resembling terminases? Mostly just a curiosity, but a one or two sentence addition clarifying this might benefit readers.

Small grammar and spelling mistakes throughout.
• Ex: Line 149 "SU10 is a Podovirdiae of the Kuravirus genus characterized by a short non-contractile tail," Podoviridae is spelled wrong.
• Genus and species names should be italicized. This is inconsistent throughout the entire manuscript.
• Line 426: "The cryo-EM of the gp73 revealed"….
• Several others places have mistakes, but I did not report them all.

Reviewer #2 (Remarks to the Author):

The authors have extensively revised this manuscript and also added some new data. They have done an excellent job of addressing my previous criticisms. The current version of the manuscript is very strong and has a number of important new insights. I have only a few minor suggestions as described below.

Abstract:
I agree with reviewer 1 that the concept of "ejection protein" versus "ejected protein" is confusing. I have worked on tailed phages for many decades (not podophages), and I did not know the definition of "ejection protein". One can assume that very few readers of Nat Comms will know what "ejection protein" means. I strongly suggest that authors avoid this term in their abstract and simply describe what these proteins do. I believe that this will make the abstract more understandable and impactful to a broader range of readers. If there were room, it might be worth mentioning that the RNAP is a massive protein that has a polymerase domain and additional domains that are involved in moving the genome into the cell.

The discussion of the HF particles is improved, but I still think it might be more conclusive. The authors say in their rebuttal to reviewer 1: "absolutely nothing is known about the assembly of Schitoviridae capsids". Experimentally this may be true, but it is safe to assume that Schitoviridae will follow the same fundamental principles seen in all other tailed phages that have been studies. With this in mind, I think one can formulate the most likely scenario for HF particle formation. HF and FF particles are the same in every way (e.g. the tail structures are the same) except for DNA content. It seems, then, that the cables could not be remnants of the phage genome remaining after DNA ejection because I don't see how DNA ejection from mature particles could occur without changing the structure of the tail. Another possibility is that HF particles result from phage DNA being completely packaged into the head, and then the DNA coming out of the head (i.e. DNA is packaged unstably in some cases-this can be seen when a head stabilizing protein is missing from some phages). Tails would have to then attach to these empty heads. Tails attaching to empty heads have not been observed in other systems to my knowledge, and there is no reason why small pieces of the phage genome would be left inside of the head in this situation. Assuming that the cables are DNA, which seems reasonable, then, the most likely explanation for the HF particles seems to be that sometimes the phage terminase packages small pieces of DNA, either fragments of the phage genome or random fragments of the host genome. If the fragments exceed some threshold size, the head will still expand, and tails can potentially attach (this has been seen in lambda). The positioning of DNA in the HF particles may be showing where the first packaged phage DNA is situated in the head. This is why similar cables are seen in FF structures. If the authors don't find this model appealing, they don't need to include it. I present it as a potential most plausible model. I do think it is worth mentioning what this DNA definitely is not (e.g. remnants of ejected DNA seems very unlikely to me even if the SU10 paper proposes this).

When I said that the reference to the SU10 cables was unhelpful, I did not meant that it should be removed from the paper. I meant that it should be explained better. The authors have now done this. Were the cables in SU10 only observed in capsids from which DNA had been ejected?

The addition of a section on the conservation of the ejection proteins is useful and informative. It can certainly be concluded that the ejection proteins are conserved among Schitoviridae. However, I do not see a justification for use of the word "ancient". This term is vague, and the

authors have not discussed the actual age of these proteins at all. They should not use this term unless they define what they mean by it (i.e. When did these proteins appear in evolutionary time? I don't think that this question can be answered).

I appreciate the addition of some extra information on the tail needle of phage P22. I do not appreciate the authors' insinuation that I should have read all the papers that they have read and know all about the tail needle without any explanation. This attitude is a real problem for these authors and explains why their paper was intitially hard to follow. Nature Comms is read by people from many different backgrounds. The authors should strive to make their work intelligible to these readers. The authors should also realize I have spent a considerable amount trying to understand this paper and make constructive suggestions for improvement. They can disagree with comments without the passive aggressive tone. Obviously, I'm not holding this against the authors as I'll be happy to see this paper published. I would just rather not be irritated while I'm writing my review.

Minor correction:
Line 493: BLASTp hit

Point-by-point response to reviewer comments
We thank the reviewers and the editor for the thoughtful review of our paper. We have done our best to address all the reviewers' comments. Below, we provide a point-by-point response: all edits are marked in red in the revised manuscript.

Reviewer #1
Generally the authors of "Integrative structural analysis of Pseudomonas phage DEV reveals a genome ejection motor" have responded well to the previous critiques and the revised paper is much better.
Thank you!

The C-terminal vRNAP binding data was an important addition to the paper, although I still find the author's conclusion that it is part of the "ejectosome" a bit of a stretch. The other proteins (gp72 and gp73) make a clear tube structure through the periplasm and resemble ejectosomes of other phages. I appreciate that the authors did a pull-down assay to show the C-terminus of gp71 interacts as the density in figure 6 is very weak and this bit of direct evidence strengthens their claims. However, regardless of it is in the same operon, and even though the gp71 protein likely associates as shown in the model in figure 9, classifying it as part of the ejectosome itself seems strange. It is ejected into the cytoplasm, but it is not spanning the periplasm like gp72/73.
There is ample evidence in the literature that phage N4 homologous vRNAP, gp50, is ejected from the virion into the host and resides in the membrane fraction [1-4]). We have added all these references to the revised paper (see **line 477**). In addition, to address the reviewer's concern, we have removed the statement that gp71 is an 'ejection protein' from the Abstract (also, see our response to Reviewer #2).

MAJOR ISSUES: • Figure 3 is hard to interpret as a static image. I requested the map and the fit of the Alpha Fold 2 model shown in 3A and this clarified a lot of points for me. So, I would suggest the authors make a movie for the supplemental data that shows a 3D rotation to accompany the figure to reduce confusion for readers.
We are unable to generate a movie at this point, but we have improved Figure 3C and its legend (***lines 1268-1278***). We believe the revised figure clearly describes how a C15 ring can generate 5 trimeric fibers.

In addition, the C15 symmetrized map should be made publicly available in the EMDB and the statistics should be added to Table 2. This is important to make available and to report the stats since major conclusions are drawn from it. In my opinion, the static figure does an injustice to the structure (not the authors' fault—it is a hard angle to show in a single figure). They would showcase their work better as a movie.
We have deposited the C15 map (EMD-45776) and the C15-symmetrized model of gp53 long tail fiber N-terminal Barrel (NTB) real-space refined against the cryo-EM density (PDB: 9COD). We report all stats in **Table 2**.

MINOR ISSUES:
Line 75: No scaffolding protein was present in the bioinformatics, but were there genes resembling terminases? Mostly just a curiosity, but a one or two sentence addition clarifying this might benefit readers.
Yes! N4 TerL and TerS were identified as gp68 and gp69, respectively, using comparative genomics. We added a statement on ***lines 77-78***. In my lab, we have solved the cryo-EM structure of gp69 (TerS).

Small grammar and spelling mistakes throughout.
• Ex: Line 149 "SU10 is a Podovirdiae of the Kuravirus genus characterized by a short non-contractile tail," Podoviridae is spelled wrong.
Fixed! Thank you.
• Genus and species names should be italicized. This is inconsistent throughout the entire manuscript.
Fixed throughout the manuscript! Thank you.
• Line 426: "The cryo-EM of the gp73 revealed"….
Fixed! Thank you.
• Several others places have mistakes, but I did not report them all.
Fixed throughout the manuscript! Thank you.

Reviewer #2

The authors have extensively revised this manuscript and also added some new data. They have done an excellent job of addressing my previous criticisms. The current version of the manuscript is very strong and has a number of important new insights. I have only a few minor suggestions as described below.

Thank you!

Abstract:

I agree with reviewer 1 that the concept of "ejection protein" versus "ejected protein" is confusing. I have worked on tailed phages for many decades (not podophages), and I did not know the definition of "ejection protein". One can assume that very few readers of Nat Comms will know what "ejection protein" means. I strongly suggest that authors avoid this term in their abstract and simply describe what these proteins do. I believe that this will make the abstract more understandable and impactful to a broader range of readers. If there were room, it might be worth mentioning that the RNAP is a massive protein that has a polymerase domain and additional domains that are involved in moving the genome into the cell.

We have removed the term 'ejection proteins' from the Abstract to follow the reviewer's suggestion.

The discussion of the HF particles is improved, but I still think it might be more conclusive. The authors say in their rebuttal to reviewer 1: "absolutely nothing is known about the assembly of Schitoviridae capsids". Experimentally this may be true, but it is safe to assume that Schitoviridae will follow the same fundamental principles seen in all other tailed phages that have been studies. With this in mind, I think one can formulate the most likely scenario for HF particle formation. HF and FF particles are the same in every way (e.g. the tail structures are the same) except for DNA content. It seems, then, that the cables could not be remnants of the phage genome remaining after DNA ejection because I don't see how DNA ejection from mature particles could occur without changing the structure of the tail. Another possibility is that HF particles result from phage DNA being completely packaged into the head, and then the DNA coming out of the head (i.e. DNA is packaged unstably in some cases-this can be seen when a head stabilizing protein is missing from some phages). Tails would have to then attach to these empty heads. Tails attaching to empty heads have not been observed in other systems to my knowledge, and there is no reason why small pieces of the phage genome would be left inside of the head in this situation. Assuming that the cables are DNA, which seems reasonable, then, the most likely explanation for the HF particles seems to be that sometimes the phage terminase packages small pieces of DNA, either fragments of the phage genome or random fragments of the host genome. If the fragments exceed some threshold size, the head will still expand, and tails can potentially attach (this has been seen in lambda). The positioning of DNA in the HF particles may be showing where the first packaged phage DNA is situated in the head. This is why similar cables are seen in FF structures. If the authors don't find this model appealing, they don't need to include it. I present it as a potential most plausible model. I do think it is worth mentioning what this DNA definitely is not (e.g. remnants of ejected DNA seems very unlikely to me even if the SU10 paper proposes this). When I said that the reference to the SU10 cables was unhelpful, I did not meant that it should be removed from the paper. I meant that it should be explained better. The authors have now done this. Were the cables in SU10 only observed in capsids from which DNA had been ejected?

We thank the reviewer for the valuable feedback. However, DEV HF particles lack the ejection protein gp72 (described in ***lines 183-184***), suggesting these particles have released their DNA (and ejection proteins). If DNA cables inside HF particles were generated by abortive packaging followed by premature tail assembly, as suggested by the reviewer, we would expect to see the ejection protein gp72 around the portal, which we do not. This assumption is based on our (limited) knowledge of ejection proteins in *Podoviridae,* where these proteins are encapsulated before genome packaging into empty procapsids. The truth is that we do not know the origin of cable DNA in DEV HF particles. Therefore, we prefer to report its phenomenological observation and refrain from speculating why/how DNA decorates the DEV capsid interior. More experimental work is needed to address the nature of cable DNA in DEV.

Regarding the SU10 paper, the reviewer's suggestion is well-taken. We now state (***lines 152-153***): "*Cable-like structures inside the SU10 prolate capsid, concentric to the portal protein, were hypothesized to be remnants of the genome left in the capsid after genome ejection*". This revision clarifies that the published work provided no hard evidence but is only a hypothesis.

The addition of a section on the conservation of the ejection proteins is useful and informative. It can certainly be concluded that the ejection proteins are conserved among Schitoviridae. However, I do not see a justification for use of the word “ancient”. This term is vague, and the authors have not discussed the actual age of these proteins at all. They should not use this term unless they define what they mean by it (i.e. When did these proteins appear in evolutionary time? I don’t think that this question can be answered).
OK! We removed the adjective ‘ancient’ from the re-revised paper.

I appreciate the addition of some extra information on the tail needle of phage P22. I do not appreciate the authors’ insinuation that I should have read all the papers that they have read and know all about the tail needle without any explanation. This attitude is a real problem for these authors and explains why their paper was intitially hard to follow. Nature Comms is read by people from many different backgrounds. The authors should strive to make their work intelligible to these readers. The authors should also realize I have spent a considerable amount trying to understand this paper and make constructive suggestions for improvement. They can disagree with comments without the passive aggressive tone. Obviously, I’m not holding this against the authors as I’ll be happy to see this paper published. I would just rather not be irritated while I’m writing my review.
We sincerely apologize to the reviewer for coming across as passive-aggressive. We swear our intention has never been to insinuate, provoke, or antagonize the reviewer. We strived to provide the most concise response to the reviewers’ feedback, backed up by new data that strengthened the paper while guiding the reviewers to see our edits without reading the entire manuscript. We realize our telegraphic responses, all in red and bolded font, may have come across as irreverent. Please accept our apologies. We immensely appreciate the time and invaluably useful comments the reviewer made about our work. We do believe the final, revised paper is much improved. Thank you!

Minor correction:
Line 493: BLASTp hit
Fixed. Thank you!

References

[1] Falco SC, Rothman-Denes LB. Bacteriophage N4-induced transcribing activities in Escherichia coli. II. Association of the N4 transcriptional apparatus with the cytoplasmic membrane. Virology. 1979;95:466-75.
[2] Falco SC, Rothman-Denes LB. Bacteriophage N4-induced transcribing activities in Escherichia coli. I. Detection and characterization in cell extracts. Virology. 1979;95:454-65.
[3] Kiino DR, Singer MS, Rothman-Denes LB. Two overlapping genes encoding membrane proteins required for bacteriophage N4 adsorption. J Bacteriol. 1993;175:7081-5.
[4] Lenneman BR, Rothman-Denes LB. Structural and biochemical investigation of bacteriophage N4-encoded RNA polymerases. Biomolecules. 2015;5:647-67.