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Reply to: About proteins of a siphophage tail tip complex reverting to their pre-ejection fold after DNA ejection

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REPLYING TO C. A. Arnaud et al. *Nature Communications* https://doi.org/10.1038/s41467-025-57874-8 (2025)

In a recent *Matters Arising*, Arnaud et al. critically reviewed our recent work on the structure of DT57C, a T5-related siphophage. Their evaluation aims to reinforce the interpretation of results by Linares et al.[1], in which the same authors present structures of T5 tails in two states: a basal state, and a state bound to its FhuA receptor inserted in a nanodisc. Our paper[2] discusses structures of entire DT57C bacteriophages in the basal, pre-ejection state (filled with DNA and the tape measure protein, TMP) and in an empty state where the DNA and the bulk of the TMP have been ejected. Thus, the combined works describe three states of the tail: pre-interaction (presented by both papers), upon interaction with the receptor in a nanodisc (presented by Linares et al.[1]), and after spontaneous DNA ejection in solution (presented by us). Importantly, no paper describes the tail of the entire virion in a state of ejected DNA while bound to the receptor.

Arnaud et al. criticize our work for apparently misleading readers to conclude that the DT57C tail tip re-closes during the host cell infection. They also claim that Linares et al.[1] provided unambiguous evidence for the expulsion of both TMP peptides (the C-terminal TMP fragment, CTMP, and the rest of it, TMP*) and conclude that our report about the presence of this fragment in the post-DNA ejection state must be an artifact. These statements, however, pose multiple problems.

Under the conditions under which our data were obtained, we did not claim that tail tip re-closing and CTMP reassociation at its initial position unequivocally take place in vivo, as was stated by Arnaud et al. Instead, we reported this effect only regarding the DNA ejection process in solution. However, based on the analysis of our structures and those presented by Linares et al.[1], we found that the baseplate hub protein (BHP) cavity lacks the space to accommodate the CTMP trimer except in a central position. Therefore, we concluded that the CTMP trimer must dissociate to bind the subunits of the open BHP in a monomeric form for the opening of the tail conduit. Such a complex mechanism would imply a specific function, and the BHP-bound CTMP would also be present in vivo after TMP* ejection. This does not necessarily imply that BHP re-closing and CTMP re-association happen in vivo, although it cannot be ruled out with currently available data. Biphasic ejection is the best hypothesis to fit possible biological functions for the only known potentially mobile protein present in the post-TMP* ejection tail conduit. However, we presented this idea clearly as a hypothesis in our paper for potential future research. In addition, we do not claim that the CTMP is the only agent leading to bi-phasic DNA ejection observed in vivo, but simply that it might be related. Therefore, our statement is not incompatible with the fact that this bi-phasic ejection is only observed in vivo and requires additional factors.

Inspection of the maps deposited by Linares et al.[1] capturing the state after FhuA receptor binding and subsequent conformational rearrangement (EMDB entries 14800 and 14873) reveals multiple less resolved regions containing unassigned map densities that may correspond to the CTMP still bound to the tail tip complex (Fig. 1). One of these unassigned regions is present in the lumen of the baseplate hub protein (BHP) (Fig. 1a, b) and appears to be of a similar length as CTMP, consistent with the interpretation of our map of the post-ejection state in which the trimeric CTMP is present (Fig. 1c). Importantly, Figure S1 from Linares et al.[1] shows only electron micrographs of negatively stained samples of tails, and Figure S3 adds a 2D class from these images. Due to the intrinsic resolution limitations and nature of the data presented in both figures, they cannot be used to determine the identity of the low-resolution density shown in Fig. 1a, b. Considering the presence of all the described unassigned densities, including the stretches of density along the inner wall of the BHP and others, it is unreasonable to conclude that CTMP was absent from the reconstructions by Linares et al.[1].

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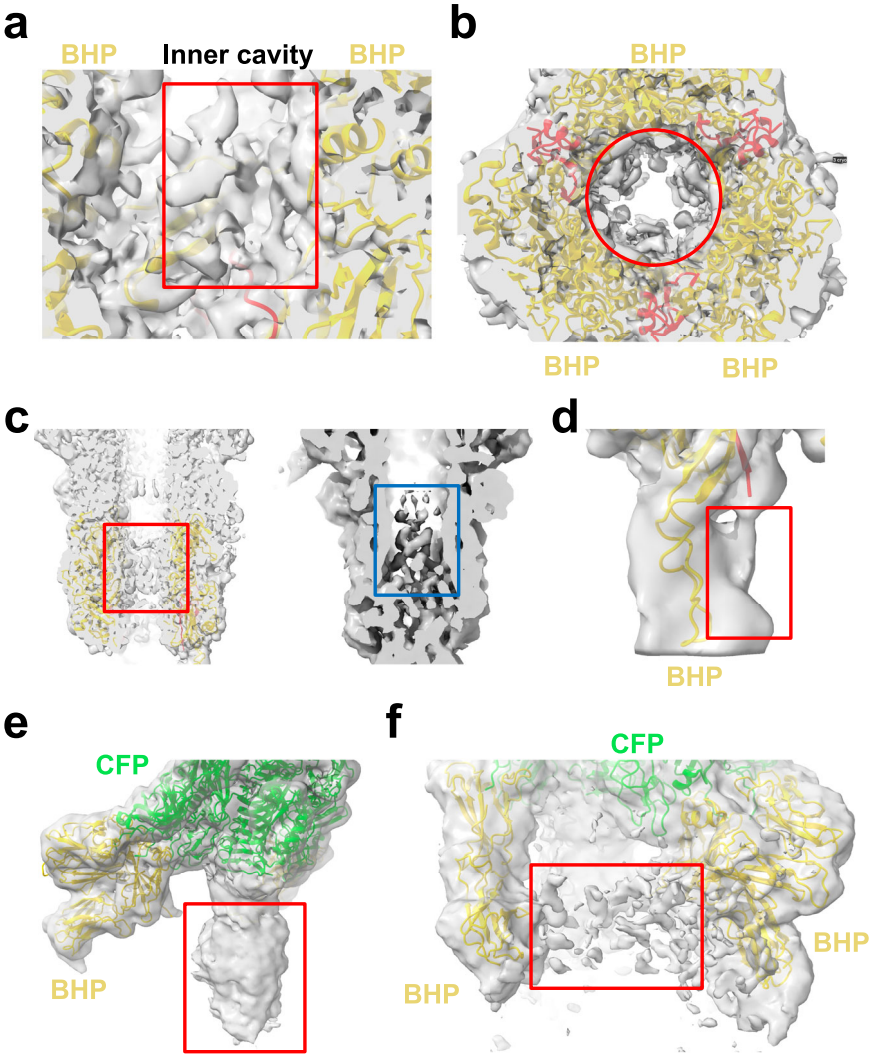

Fig. 1 | Unassigned density regions in the reconstructions by Linares et al. contain features consistent with fragments of TMP. a Map features located along the inner wall of the baseplate hub protein (BHP) in EMD-14873. EMD-14873 is displayed after applying a local resolution filter calculated from the deposited half maps. The location of this density region coincides with that of the C-terminal fragment of the TMP (CTMP) in our map. **b** Radial section of the same area as in (**a**). The unassigned densities can be seen parallel to the inner wall of BHP. **c** Comparison of EMD-14873 (left) and our reconstruction of the empty bacteriophages (right). Red box: unassigned region of density shown in (**a**) and (**b**). Blue box: region in our empty bacteriophage reconstruction corresponding to CTMP. **d** Additional unassigned region with low-resolution density is present in EMD-14873. **e** Region of unassigned density in EMD-14800. Due to this region's low resolution, it is impossible to determine what component it contains. Therefore, it cannot be ruled out that it includes the CTMP after conformational rearrangement upon binding to the FhuA receptor. The deposited unsharpened version of EMD-14800 is displayed. **f** Additional region with weak density observed in EMD-14800.

Arnaud et al. also claim that our empty phage structure was an artifact. This claim is not supported by any evidence. On the contrary, we separated particles of full and empty virions and selected their corresponding tail tips in individual classes. All the particles from empty virions had an empty capsid and lacked TMP within the tail tube lumen. We can confidently conclude that these originate from native phages that have ejected their DNA, as explained below.

One of the reviewers considering our paper raised the possibility that empty phages might have originated by DNA escape through breakage points in the capsid. This possibility was ruled out by additional analysis of nine deposited tomograms (not a single one, as claimed by Arnaud et al. See "Data availability"), each containing multiple phages (full and empty). The majority of empty phages are not bound to any vesicles, as seen in EMDB entries 37518, 37519, 37539, and 37543. Although it is indeed plausible that these vesicles, remnants of host cellular membranes, could have triggered DNA ejection in some virions, leading to the observed empty phages, in the absence of direct evidence for the involvement of these vesicles, we chose not to discuss this topic further. Importantly, our conclusions were independent of factors that may have triggered the DNA ejection in a fraction of the particles in our samples. Empty-tailed virus particles cannot be assembled by aberrant morphogenesis. This would directly contradict the well-established morphogenetic pathway of T5-like phages[3–5], and it additionally would ignore the fact that empty particles pre-existing the gradient centrifugation would be removed during purification. Arnauld et al. also suggest that the missing wedge (an artifact of single-axis tomographic reconstructions that leads to anisotropic resolution) might have hidden capsid breakage points in the empty virions observed in our tomograms. Nevertheless, this is extremely unlikely, as it would require perfect alignment of these breakage points within the missing wedge in all 12 tomograms of empty virions with intact capsids we presented. Finally, Arnauld et al. grasp at straws in their *Matters Arising* by raising the temporal and reversible weakening of the head-to-tail joint as a new possibility, allowing the release of the DNA and TMP*. However, this is purely speculative and not supported—even indirectly—by any experimental

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evidence or structural model. Furthermore, our structure of the DT57C head-to-tail interface revealed a solid attachment of the tail completion protein into the head completion protein without indicating that a hypothetical reversible opening of the head-to-tail joint could occur.

We do not see an explicit need for a specific energy source to explain CTMP reassociation after DNA ejection into the solution. Protein folding is generally an exothermic process. It is rather more likely that an energy source was required for the endothermic dissociation of the trimer and to open the tip complex. That energy could be provided by interactions with the cell surface or by the energy from releasing the DNA packed at a very high density in the phage capsid. Regardless, no available data indicate a potential gap in the energy balance of the tip transitions. Therefore, we do not deem it necessary to analyze this problem in depth further here.

Regarding our deposited central tail fiber (CFP) model, we are aware of the limited resolution of the corresponding reconstruction. For these reasons, we clearly stated in our paper's Results section that we fitted a predicted model in that region. Furthermore, for the same reasons, we did not perform a detailed analysis of the CFP structure beyond a general description of its overall arrangement. We agree that there is indeed a need for a standardized procedure for such data. However, since no guidelines are currently established, this point is irrelevant, as it does not affect the soundness of our conclusions nor the quality of the presentation of our data. Critically, the origin and limitations of the model were disclosed entirely and taken into consideration.

It is worth noting that Linares et al.[1] only observe the side domains of the CFP folded back compactly over the DII regions in their reconstruction in the receptor-bound state without supporting data that this architecture was adopted in the basal state. The composite atomic model they now present (Fig. 2c of Arnaud et al.) can, therefore, not be deemed a representation of the actual structure of the pre-ejection state.

We recognize the significance of all the publications mentioned by Linares et al.[1] Nevertheless, due to space limitations, we chose to cite and discuss only those directly relevant to the presented findings in our paper. A separate, up-to-date review would be appropriate for a comprehensive literature discussion providing any structural information about T5-like viruses.

We thank Arnaud et al. for the opportunity to put our work in context with their findings and previous literature, and we hope to have done so as much as possible, given the limitations of this format. Considering the inconsistencies between their model (which they deem incompatible with our data) and previous literature, we would also like to invite them to perform a similar exercise. For example, based on mass spectrometry experiments with and without trypsinolysis, Linares et al.[1] assign the cleavage point in the TMP after R1127. However, this directly contradicts previous work[6] claiming that "N-terminal sequencing and mass spectrometry (MALDI-TOF) of Pb2 isolated from T5 ghosts led us to conclude that maturation was at the C-terminal end, resulting in a polypeptide of 123.9 ± 0.1 kDa (cleavage site after Val1148 ± 1) (data not shown)." We understand that methodological and instrumental advances have been made since reporting the TMP cleavage site at V1148, as in all disciplines. Nevertheless, this does not invalidate the results obtained with previous experimental techniques, especially the N-terminal sequencing data. Indeed, the uncertainty in the V1148 cleavage site was reported to be ±1 residue, which would make this position still very distant from the new cleavage site (R1127) reported by Linares et al.[1], even in the most favorable case. We note that R1127 coincides with a trypsin cleavage site, making the peptide mapping presented in Figure S5G of Linares et al.[1] less informative about the native cleavage site of the TMP. Nevertheless, neither raw data of the top-down proteomic experiment by Linares et al.[1] nor of the MALDI-TOF and N-terminal sequencing by Boulanger et al. were made publicly available.

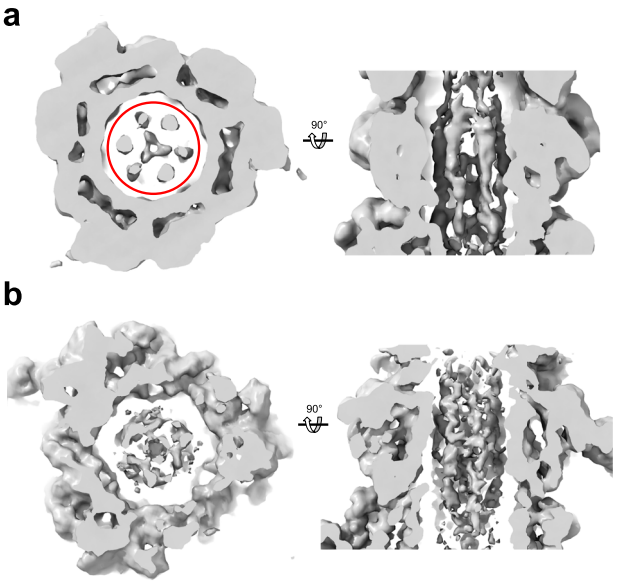

Fig. 2 | Presence of a hexameric structure in the lumen of the tail tube. a Left: cross-section at the second tail tube protein ring level from the tail tip in our reconstruction of pre-ejection bacteriophages (EMD-34955). Right: side view of the same region. The unsharpened reconstruction is shown. The hexameric structure is consistent with previous literature indicating that six copies of the bulk of the TMP are present in each phage. **b** Same region as in (**a**) but showing map EMD-14869 by Linares et al., who dismissed the possibility of six copies of the large TMP fragment in the tail tube of purified T5 tails.

Therefore, in light of the apparent contradiction between these two studies, further investigation is needed to determine the native TMP cleavage site unequivocally.

Linares et al.[1] also proposed a model for the system's state after the complete ejection of the bulk of the TMP from the tail (which corresponds to the instant immediately following the state observed in their FhuA-bound reconstruction). In this hypothesis, the bulk of the TMP remains in a hinge-like conformation with its C-terminal 43 residues bound to BHP and the rest up to its N-terminus pointing away from the tail tube (Figure 5E of Linares et al.[1]). This, however, poses a significant topological problem, as it would require the movement of the TMP through the opened BHP channel in the state of a sliding hairpin to transition from the initial linear arrangement with the N-terminus located at the opposite end of the tail tube, proximal to the capsid. Unfortunately, this point was overlooked by Linares et al.[1] in the interpretation of their results. We are glad that in the revised version of their *Matters Arising*, Arnauld et al. have taken the problem of the TMP* topology highlighted by us into account by introducing an "inverted sleeve" model. This proposed mechanism should be further verified theoretically and experimentally. Among other tests, the compatibility of the TMP sequence over its entire length with the movement in the form of a sliding hairpin should be addressed in future research.

Arnauld et al. conclude that only three copies of the entire TMP are present in T5-like phages because they only observe three copies of a fragment. This is, however, not necessarily the case. In fact, upon closer inspection of our deposited entry EMD 34955, we identified a hexameric structure in the tail tube lumen at the level of the second tail tube ring (Fig. 2a). While a three-fold structure (different from the trimeric structure corresponding to CTMP) is present inside the main hexameric structure in the tail tube lumen, it only appears in the center of the tail tube. It spans a very narrow length; therefore, this three-fold structure does not represent the stoichiometric state of the bulk of the TMP along the tail tube. Together with prior findings that TMP is present at a stoichiometry of 5 or 6 copies per phage[7], this indicates

that while only three copies of the C-terminal fragment are present, six copies of the bulk of the TMP may be found in each phage. It is unclear whether the reconstruction by Linares et al.[1] contains this hexameric structure (Fig. 2b). While we agree that the mentioned rough protein abundances calculated from band densitometry are not accurate enough to determine the actual stoichiometry of each virion component on its own, it certainly adds to the combined evidence pointing towards six copies of the bulk of the TMP. Noteworthily, an arrangement similar to the one we describe, with six copies of the bulk of the TMP but only three of its C-terminal parts, has been recently described in *Anabaena* myophage A-1(L)[8]. The exact TMP stoichiometry and its possible implication for resolving the apparent contradiction between our data and the model by Linares et al.[1] should be addressed in future experimental work.

In conclusion, our results do not contradict any data presented by Linares et al.[1] Instead, in light of all the currently available data, our findings indicate that some parts of their proposed mechanistic model may require to be updated. We hope that the phage community will take this exchange as an initiative to investigate the structural basis of the infection process in siphophages further.

Data availability

Previously published cryo-EM maps and the nine tomograms discussed here are available online from the Electron Microscopy Data Bank (EMDB) under accession codes EMD-37518, EMD-37519, EMD-37521, EMD-37531, EMD-37534, EMD-37536, EMD-37539, EMD-37543, EMD-37544, EMD-14800, EMD-14869 and EMD-14873.

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Author contributions

A.V.L, O.S.S., and M.W.: conceptualization, design of the experiments and supervision. O.S.S. and M.W.: funding acquisition. E.E.K., A.K.G., and A.V.L.: phage propagation and purification. R.A. and T-H.C.: cryo-EM data collection. R.A., T-H.C., A.V.M., M.W., and M.A.S.: data processing. P.S.O., R.A., and M.A.S.: molecular modeling. R.A., A.V.L., T-H.C., A.V.M., O.S.S., and M.W.: writing manuscript. R.A. prepared the figures. All authors contributed to interpreting the data and writing the manuscript.

Competing interests

The authors declare no competing interests.

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