nature communications

Matters arising

https://doi.org/10.1038/s41467-025-57874-8

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

Received: 21 March 2024

Accepted: 27 February 2025

Published online: 25 March 2025

Check for updates

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ARISING FROM R. Ayala et al. *Nature Communications* https://doi.org/10.1038/s41467-023-43824-9 (2023)

Electron cryo-microscopy (cryo-EM) has become a leading tool of structural biology, thanks to direct electron detectors and rapid progress in image analysis. Its impact on structural studies of bacterial viruses, or bacteriophages, is especially prominent as it is the only technique that allows the determination of the structure of large macromolecular complexes such as viral particles to atomic or near-atomic resolution. Siphophages, characterised by a long flexible tail attached to the icosahedral DNA-containing capsid, remain understudied despite being the most common among known bacteriophages (phages)[1]. The mechanism whereby the siphophage tail initiates infection is poorly understood, unlike that of the contraction of the myophage tail[2,3]. A model based on recent structural data and which is consistent with known biochemical observations and thermodynamic considerations was recently proposed by a majority of the authors of the present letter, after the determination of the cryo-EM structure of siphohage T5 tail before and after interaction with its receptor[4].

In a recent article, Ayala et al.[5] provide a structural analysis of DT57C, a T5-like siphophage also infecting *Escherichia coli*. Their cryo-EM structures are very well complemented by molecular dynamics data. However, we intend to challenge this publication. In our opinion, the presentation of some of the data, interpretation in the biological context, and comparison with existing work (most notably with the very closely related phage T5), as well as some introductory statements, are inaccurate and/or contradict the state of knowledge without clearly establishing a rational for these discrepancies.

In the abstract, it is written that "The presence of the C-terminal fragment of the TMP (TMP_{Cter}) that remains within the tail tip suggests that the tail tip complex returns to its original state after DNA ejection"[5]. This statement, then detailed in the paper, has strong implications for the mechanism of DNA ejection and cell wall perforation by the phage. However, it seems inconsistent with data available in the literature[4]. Indeed, incubation of virions with detergent-solubilised receptors systematically results in empty phages with an open tail tube and the disappearance of the straight fibre[6]. Furthermore, Linares et al. provide phage T5 tail tip structures before and after interaction with the phage receptor embedded in a nanodisc, *i.e.* close to what happens in vivo. It clearly shows (i) the total expulsion of TMP_{Cter} and the partial expulsion of the other, large TMP fragment, TMP*, (ii) the opening of the baseplate hub protein (BHP), (iii) the anchoring of TMP* in the BHP and its insertion in the membrane to form a channel and (iv) the bending of the straight fibre. These dramatic conformational changes are clearly incompatible with reversibility (Fig. 1a, b).

Ayala et al. conclusions are based on the structure of the 20% empty phages present in the sample[5]. Such a high proportion of empty phages in a purified phage sample is surprising. The resulting empty structures do not stem from DNA expulsion after interaction with the receptor. Ayala et al. present tomograms of empty phages and suggest that DNA could only have been ejected through the tail tip[5], but those empty phages are not characterised biologically nor biochemically, and their relevance as an intermediate in the infection process is not established. The tomograms of empty phages are indeed beautiful. However, with the tomographic missing wedge, capsids are not fully resolved. It is thus difficult to conclude that they are perfectly intact. Another hypothesis explaining the emptying of the phage would be that the head-to-tail joint weakened partially and temporarily, allowing the expulsion of both DNA and TMP*. The structure after interaction with the receptor[4], solved from an in vitro but well-characterised sample, much more likely corresponds to an intermediate in the infection process: the TMP_{Cter} is ejected from the tail tip and TMP* partially inserted in the nanodisc lipid bilayer[4]. To support their hypothesis of TMP_{Cter} remaining in the tail tube, Ayala et al. reanalysed the map of Linares et al. [4]. They suggest that the TMP_{Cter} is also present, in unattributed densities in our structure. As discussed in Linares et al., these densities *unambiguously* correspond to TMP* that has not been completely expelled from the tail, as very clearly seen in the

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https://doi.org/10.1038/s41467-025-57874-8

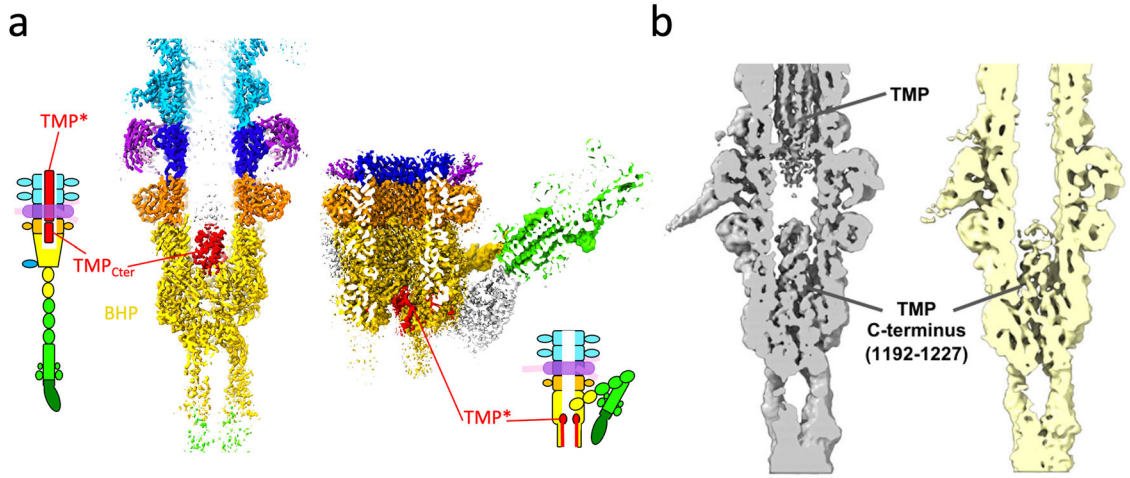

Fig. 1 | Comparison of the cryo-EM structures of the tail tip complex of phages T5 and DT57C. a Tail tip of phage T5 before (left) and after (right) interaction with the receptor[4] and **b** of phage DT57C from full (left) or empty (right) tails (from Fig 6 [5]). In **a**, **b**, the presence of the TMP and TMP$_{Cter}$ are indicated.

negative stain (Fig. S1F, white arrow of Linares et al.[4]) and in a low-resolution section of the cryo-EM map (Fig. S3C of Linares et al.[4]). Attribution of this density to TMP$_{Cter}$ is highly improbable as it would mean monomerization of the protein to fit in these separated densities (Fig. 1a–c of Ayala et al.[7]). Other "unassigned" densities pinpointed by Ayala et al.[7] (Fig. 1d, e) are clearly attributed in Linares et al.[4] to TMP*, RBP$_{pb5}$, respectively. It is not clear what is boxed in Fig. 1f[7].

Another controversy of Ayala et al. article is the source of energy needed for the complex to revert to its original state. In the mechanism discussed in Linares et al., the energy required to open the tail tube and perforate the cell wall would come from the transition of the different tail tip proteins from a metastable conformation to a more stable conformation, induced by receptor binding. This mechanism is detailed at the molecular level[4,8]. Indeed, it occurs even in the minimal system of purified tails incubated with receptors. Thus, this mechanism is independent of the presence of—under pressure—DNA-filled capsids or other host factors. A similar mechanism, relying on protein conformational change to a thermodynamically more stable fold after receptor binding, has also been proposed for podophage T7[9] as well as for the contraction of the myophage tails[2,3] and to our knowledge, it has never been proposed nor shown that they retro-convert to pre-injection state. In the case of T5 and T5-like phages, a reversible mechanism would imply refolding of several proteins, including also dissociation of TMP* from the BHP, extraction of the BHP from the outer-membrane outer leaflet, reclosure of the BHP, re-entering of the TMP$_{Cter}$ in the BHP and unbending of the straight fibre. Overall, the reversible mechanism described by Ayala et al. seems not supported by data and lacks the required energy. Ayala et al. further suggest that TMP$_{Cter}$ could be involved in the biphasic DNA ejection observed in the T5-like phages. However, this biphasic ejection only occurs in vivo. Thus, this hypothesis also lacks a structural basis.

Ayala et al. propose an atomic model for the Central Tail Fibre Protein (PDB 8HRE). Their map in this region of the tail tip is poorly resolved (overall resolution 5.5 Å), with resolution dropping to more than 10 Å towards its extremity[5] (Fig. 2a). As described in the methods section in Ayala et al.[5], the proposed model was initially obtained using AlphaFold2. However, with the poor experimental data in this region of the map, it is debatable whether this model should be branded as a bona fide structure deposited in the PDB (Fig. 2). Rather, Supplementary Fig. 23 in Ayala et al.[5] should contain AlphaFold2 confidence levels and the legend should state that this is not based on, nor confirmed by, experimental data. Indeed, at variance with the AlphaFold2 model, Linares et al. show that the extremity of this protein folds as a continuous ß-helix, decorated with two side domains (Fig. 2d, e), and is capped by the receptor binding protein[4,8]. Thus, the model 8HRE deposited in the PDB is not supported by experimental data and is wrong, as known from data from the literature[4] (Fig. 2).

In the Ayala et al. abstract, it is written, "Considerable portions of T5-like phages remain structurally uncharacterised"[5]. Phage T5, indeed included in the archetypical T-series of model phages at the beginning of the XXth century, has been largely studied and structural data has been accumulated over the years. To mention only atomic structures available, these include the structures of the Distal Tail Protein[10] (PDB 4JMQ), the C-terminus of the Lateral Tail Fibres[11] (PDB 4UW7, 4UW8, 5AQ5), the decoration protein[12] (PDB 5TJT, 5LXK), the Tail Tube Protein[13] (PDB 5NGJ), the capsid in different maturation conformations[14] (PDB 6OKB, 6OMA, 6OMC), the whole tail tip before and after interaction with its bacterial receptor[4] (PDB 7QG9, 7ZHJ, 7ZLV, 7ZN2, 7ZN4, 7ZQB, 7ZQP), the Tail Terminator (PDB 8BCP, 8BCU) and the Receptor Binding Protein bound to its receptor[8,15] (PDB 8A8C, 8B14, 8A60), *i.e.* nearly all parts of the phage. The different structures are published, deposited in public data banks and freely available. Thus, Ayala et al. do not fully recognise the work done over the years by many scientists. Only three papers on this long list are cited in Ayala et al.[4,13,14]. The authors also state, "A unique feature of DT57C is the mode of attachment of the lateral tail fibres to its tail tip"[5]. This mode of attachment, although not detailed at atomic resolution, was already pointed out in T5[4].

Finally, the title of the Ayala et al. article is "Nearly complete structure of bacteriophage DT57C reveals architecture of head-to-tail interface and lateral tail fibres"[5]. Residues 8–54 or so of the fibre protein are traced. This represents less than 5% of the full-length protein (1076 residues). Thus, the "nearly complete" disclosure of the architecture of the lateral fibre seems largely overstated.

In their reply to our Matters Arising, Ayala et al. question some of the results presented in our Linares et al. article[4]. Here are answers to the different points raised:

Position of the TMP cleavage site—In Linares et al., LC–ESI–TOF–MS on whole tails, *i.e. without* trypsin digestion, was used to determine the exact mass of all proteins of T5 tail[4]. For the TMP, masses of 121,683 and 9702 were obtained, to be compared with theoretical masses of 121,681 and 9702, which results in protein cleavage after R1127 (all detailed in Fig. S8C of Linares et al.[4]). Furthermore, Boulanger et al.[16], who propose another cleavage site, used solely MALDI, and *not* N-terminal sequencing, a technique which is less accurate than ESI–TOF. Instrumentation also made a lot of progress in 15 years, and therefore, even if they contradict old data, we are confident in our results.

https://doi.org/10.1038/s41467-025-57874-8

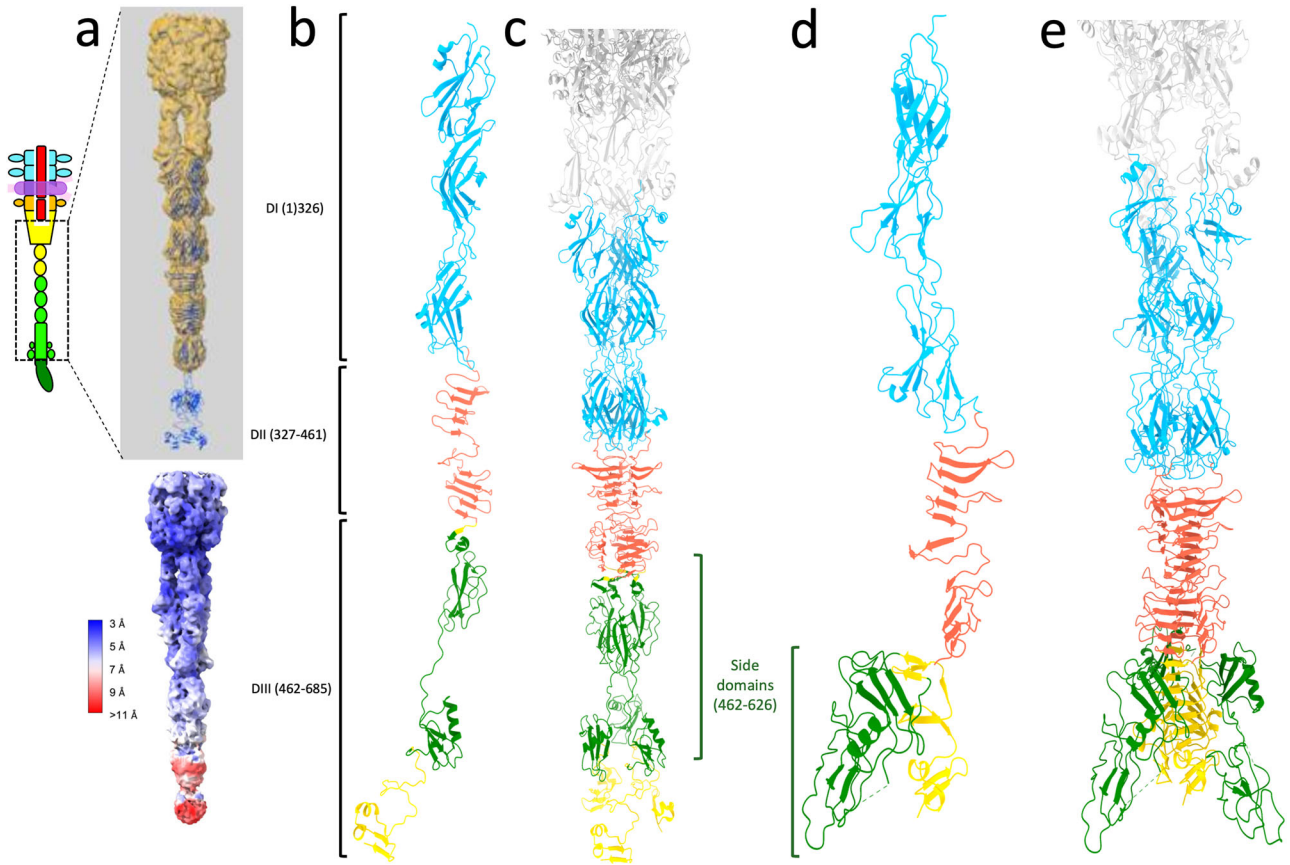

Fig. 2 | Central tail fibre protein structure. a Map-model overlay of the PDB validation report of entry 8HRE (top) and local resolution map of phage DT57C central tail protein (bottom, from Fig. S28[5]). **b**, **c** Ribbon representation of the structure of the central tail fibre protein of phage DT57C (PDB 8HRE, inspired from Fig. 23[5]) and **d**, **e** of phage T5 (PDB 7ZLV)[4]. These two proteins share 77% identity and 87% similarity. In **b**–**e**, residues 1–326 are coloured blue, 327–461 in orange, 466–626 in green and 462–465, 627–689 in gold. In **c**, **d**, the side domains (residues 466–626, in green) are indicated. As stated in Linares et al.[4] these side domains are only resolved in the central fibre map after interaction with the receptor because they are stabilised in that conformation. **d**, **e** Thus represent a composite structure. The density for the central 24-stranded ß-helix (orange and gold in ((**e**)) is, however, very clearly defined in the density of the purified tails.

Topology of TMP* after the tail interaction with the receptor—as mentioned in Linares et al.[4], the resolution in the area of the map connecting the tail tube to the nanodisc is very low, thus we considered it questionable to elaborate further about the channel that we propose inserts in the nanodisc and only presented the available raw data. The structure of T5 tail tip after interaction with FhuA is an intermediate of TMP ejection (see Figs. S1F and S3C4 of Linares et al.[4]). This structure includes 43 residues of the C-terminus of TMP* anchored between two subunits of the BHP. As pointed out by Ayala et al. in Fig. 1d[7], the density continues towards the membrane, merging with the BHP 'legs' that plunge into the nanodisc. As discussed in Linares et al.[4], TMP* would thus be insert into the outer-membrane, a stretch of 46 hydrophobic residues being predicted at this position (Fig. S5G[4]). Depending on the length of the transmembrane helices, this sequence could span two or three times the nanodisc/outer membrane. In the intermediate state of Linares et al. shown in Fig. S3C[4], there is clear density in the lumen of the channel of the tail tube, suggesting that TMP* coiled-coil exits through the channel, as when a sock or a sleeve is inverted. Clearly, the resolution is not good enough to discuss more, but there does not appear to be a "significant topology problem", as suggested by Ayala et al.[7].

Stoichiometry of the TMP—unlike their conclusion in Ayala et al.[5], Ayala et al.[7] now propose the TMP to be a hexamer. We resolved a TMP_{Cter} trimer in the T5 tail tip and three copies of TMP* were inserted between the three BHP subunits after interaction with the receptor. A TMP_{Cter} trimer was also resolved in the tail tips of siphophages 80α[17], λ[18,19], R4C[20] and JBD30[21]. It might be that there is a TMP* hexamer further up in the tail, but we have not investigated it, and as we observe a trimer of both TMP_{Cter} and TMP*, it would be counter-intuitive to conclude otherwise.

Data availability

PBD files mentioned in this letter are as follows: 4JMQ, 5TJT, 5LXK, 4UW8, 4UW7, 5AQ5, 6OKB, 6OMA, 6OMC, 7QG9, 7ZHJ, 7ZLV, 7ZN2, 7ZN4, 7ZQB, 7ZQP, 8BCP, 8BCU, 8A8C, 8B14, 8A60, 8HRE.

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Acknowledgements

We acknowledge funding from the Agence Nationale pour la Recherche ANR-20-CE11-0023 (C.B. and G.S.). Our work uses the platforms of the Grenoble Instruct-ERIC Centre (ISBG; UAR 3518 CNRS-CEA-UGA-EMBL) within the Grenoble Partnership for Structural Biology (PSB), supported by FRISBI and GRAL, financed within the University Grenoble Alpes graduate school (Ecoles Universitaires de Recherche) CBH-EUR-GS. The IBS acknowledges integration into the Interdisciplinary Research Institute of Grenoble (IRIG, CEA).

Author contributions

C.A.A., R.L., O.R., E.B.E., P.B., G.S. and C.B. contributed to the analysis and interpretation of the data. C.B. made the figures and wrote the text. C.A.A., R.L., O.R., E.B.E., P.B. and G.S. proofread the text and approved of it.

Competing interests

The authors declare no competing interests.

Additional information

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