

# Long read metagenomics reveals phage dynamics in the human gut microbiome

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

This manuscript describes a large scale metagenomics study of prophages in the human gut microbiome. Its key originality is that it uses long read sequencing (and also short read for comparisons and controls) which results in higher accuracy because long reads allow more complete genomes and facilitate the identification of the host (by linking the prophage with its genomic context). The study uses two time points (2 years apart) in 6 human healthy individuals. First, it confirms that long-read sequencing provides fewer and much longer phage genome sequences. It also correctly identifies a much larger fraction of phages as integrated (phage-plasmids being usual a minority). This allows to compare three standard prophage identification tools, finding that Genomad seems superior to the others. Once this more technical part is done with, the study makes 5 major claims.

- Prophages are usually stable within their hosts over a 2-year time frame. This fits and complements very recent data from the Good lab using short read sequencing.
- Lysogens and naive hosts can co-exist in a population at varying concentrations. I found this results to be of particularly interest. Several preprints have been revealing patterns of phage-bacteria co-occurrence that do not seem to fit with the classical lysis-or-lysogeny model. This study provides an explanation for it, by showing that among otherwise identical bacteria only some carry the prophage.
- Phage induction occurs predominantly at low levels. This differs from other studies where under perturbation one observes phage bursts. This is a very important finding, especially in combination with the previous point, because it shows what is occurring under normal conditions.
- Phages can infect bacterial hosts of different taxonomic families. The observation that some phages can infect distant hosts is interesting, but is less novel than the rest. This is well known both for ssDNA and dsDNA phages.
- Some prophages may have domesticated insertion sequences instead of using the typical integrases. Phages using transposases have been known for decades, but here the authors identify a novel intriguing family.

In short, this manuscript demonstrates the interest of using long-read sequencing for phage metagenomics. It also provides some exciting novel data on the dynamics of prophages and their induction in the gut. Finally, it extends our knowledge on temperate phage host range and discovers a novel type of transposase-driven integrative temperate phage. The manuscript is clear and well-written. I believe this may become a landmark study in the field.

Some comments/suggestions.

- It would be pertinent to comment figure 1b in relation to the relatively low fraction of integrated regions. 60% seems well below the frequency of integrative prophages among prophages. A discussion on how many of the remaining 40% are not integrative prophages and how many are poorly covered integrative prophages would be useful.
- Line 206. One needs an histogram of the number of prophages per cluster (to see how balanced it is this distribution) in supplementary material to better understand this result. The other (more important) reason for this plot is related to the statements in line 216 where is indicated that 5% of the clusters were gained or lost. It was not completely clear to me if this concerns the number of phages or clusters (and whether that mattered).
- It would be relevant to discuss if these prophages could have been present in the other time point at low frequency and therefore been missed. Some statistics would help too: Are these prophages among the rarest or the most frequent (when present?). And are their hosts changing a lot in frequency? This is a very interesting result that requires some further details.

- A potential confounding variable is the detection of the strain. One needs more details on what was done to ensure that the strains at T1 and T2 are the same. If in a certain percentage of cases they are closely related strains, then this would contribute to explain the 5% of gained or lost prophages.
- If there is an heterogeneity in the presence of prophages in a population, some of the 5% of gains/losses actually result from variants that never got infected by the prophage. This interaction between the two factors (non-ubiquity at a time point and gain/loss between time points) needs to be quantified.
- Insertions and deletions are measured by using the average coverage. While this is sometimes rather obvious (fig 2b), the data on heterogeneity in the population is less trivial. For example, in figure 2d there is a lot of variance outside the prophage region (and I assume other cases are even more subtle). As a result, one needs more substantial statistical evidence that the existence of coverage in the prophage region is not the result of such variations. If the page was completely absent, then no reads should have been obtained, but given the variance one needs some information on the intervals of confidence for the % of presence of the prophage. In the same vein, the comparisons between the sniffles analysis and Genomad, seems to assume the previous is absolutely correct, which needs characterisation/justification.
- Line 469. Interesting hypothesis, but it was not clear to me how this could work. I agree that induction requires functional prophages. But since there is super-infection exclusion these phages would go nowhere if the bacteria in the vicinity are lysogens even if partially defective (except if defect concerns specifically the exclusion mechanisms). This could be some sort of bet hedging, where a small population excises to “explore” the possibility that some sensitive cells are present in the population where the majority remains in the existing favourable host. This would fit the data showing that lysogens and non-lysogens co-exist.
- I had some difficulties with Figure 3. Occasionally, phage regions (green rectangles) are smaller than the conserved region. I guess this is Genomad getting edges wrong. But in panel d, one wonders what is the real structure in terms of what is between the two rectangles (each is too small to be a prophage) and what is the third region of similarity (a satellite?). It doesn't fit the narrative about integration/excision that coverage does not match the prophage prediction (which is smaller) neither the homologous region (which is larger). I also find surprising that the regions around the prophage are always non-homologous. This seems more expected in panels d and e (different species), but not in a. This example would be more clear if one could have in supplementary material a figure with gene names/functions.

#### Minor comments.

- Line 55. I would suggest to introduce Cre differently, because implicitly the text suggests it's an integrase of an integrative phage, when it's a resolvase of P1 (as stated later in the text).
- Figure S1, panel E. The authors should check the underlying data. The three box plots seem strictly identical, which seems really unlikely. There may have been a problem in plotting the data.
- Line 284 and discussion. Beyond metagenomic studies, the existence of phages that were experimentally shown to transfer across genus had been known for a while (PR5, P1 or PRD1). The quantification here done is interesting, nevertheless.
- While I appreciate the name IScream phages I found the use of Cre in the name misleading, since Cre is a tyrosine recombinase.
- Line 472. Not clear to me which data suggests that phages have evolved to be more specific and narrow host range (suggesting this is a macro-evolutionary trend). I'm afraid I don't get that from the cited paper. The precise meaning of the statement should be clarified.
- The Lopez et al preprint is now published.
- Some references lack page numbers or ids: 30, 35, 71 or have irrelevant text : 15

(Remarks on code availability)

#### Referee #2

##### (Remarks to the Author)

Wirbel et al., in “Discovering...”, present a study of phages in the gut microbiome using long read sequencing. Twelve samples were studied from six healthy individuals studied over two years. The authors find that phage populations are generally stable over time. Sometimes host bacteria exist as mixed populations where some but not all members of the species contain specific integrated prophages. A few phages were found to infect multiple hosts. A lineages of phages (IScream) was identified that was proposed to use IS30 machinery for integration. Follow up studies suggested that the IScream sequences appeared in virus-like particles released from cells.

The long read sequencing method is useful for documenting phage genomes comprehensively. Some of the points on phage dynamics have been reported previously or anticipated in other work, such as longitudinal stability and low levels of induction in gut. Still, this study presents a useful overview with improving technology, and adds some interesting points, such as observing bacterial strains with and without specific prophage, and the finding that a few human gut phage seem to have broad host ranges.

The demonstration of naturally occurring phages that use IS30 machinery for site specific integration is novel and interesting. Previously phage Mu was shown to use a DDE-type enzyme for transposition to allow genome multiplication prior to packaging; IS30 transposase is also predicted to be a DDE-type enzyme. IS30 was also shown to be able to substitute for lambda integrase to support bacteriophage replication (ref 41). Still, it is quite interesting to see the apparent wide use of this mechanism documented in metagenomic data.



Minor points.

In the Discussion the authors write: "Previous studies investigating human gut virome dynamics, based on short-read VLP sequencing during a period of up to a year or using bulk metagenomic sequencing over shorter time frames (10 days), have reported generally high stability and individuality of the virome<sup>20,27,48</sup>." Actually reference 20 describes sampling over 2.5 years analyzed by metagenomic sequencing.

Given that it is possible to grow an IS30-using phage, maybe it would be nice to include a particle EM image?

(Remarks on code availability)

Referee #3

(Remarks to the Author)

Wirbel, Hickey et al, provide a useful look at phages in long read gut metagenome assemblies. Using very creative bioinformatics analyses, they've uncovered new biology and provide a strong case that existing virome tools can be improved, especially when it comes to prophage boundary identification.

I found both the data and methodologies to be quite compelling. Soon the metagenomics field will be awash in long read-based metagenomic assemblies, and this manuscript helps lay the groundwork for understanding these data.

Critiques and questions:

The authors state that prophage dynamics are low (~5%). However, since only 35% of bacterial chromosomes can be recovered between samples, perhaps the fraction is more like (5%/35% = ~14%). Please comment on this.

Assembly errors still occur in long read-based assemblies. This gives me some skepticism over claims that some prophages are able to integrate in bacteria from other families. For phages mentioned in Fig 3, S7, and S8, can you do due diligence to those assemblies. Ideas include using another assembler (myloasm?) or checking that the phage region read methylation calls match the rest of the (host) contig.

On the other hand, you state in line 472 "It is important to note, though, that lysogenic host range does not necessarily imply productive host range (the ability to produce infective particles from multiple hosts)". If you perform read analysis for induction (circularity, excision), this would provide compelling evidence for productive infection. I understand that reads implying circularity and excision are not detectable in all prophage regions.

Are prophage regions with evidence of excision/circularity more (or less) likely to be dynamically gained/lost?

The prophage boundary analyses, using long read data as ground truth are particularly compelling and important. These and related datasets can help train the next generation of phage/prophage tools to better identify boundaries. Having said this, please add CheckV and Cenote-Taker 3 performance to the analyses related to Fig S6.

Relatedly, the Virsorter2 SOP suggests using CheckV for prophage boundary determination:  
<https://www.protocols.io/view/viral-sequence-identification-sop-with-virsorter2-5qpvojqebg4o/v3>

Fig 4B: why is the IS element only annotated on the left end of the phage? Fig 4A and D indicate that there should be a truncated IS element on the right side.

Analysis repos for the manuscript are not publicly available! Please ameliorate this!

- <https://doi.org/10.5281/zenodo.15192469>

- [https://github.com/bhattlab/long\\_read\\_benchmark](https://github.com/bhattlab/long_read_benchmark)

(Remarks on code availability)

As noted above, the code/data repos are not publicly available.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors took all my comments seriously and answered them in 16 pages with numerous text changes and novel data

and figures. I think the manuscript has improved and I have no further comments. I'm ok with the new name IScream as it is not explicitly mentioning Cre as a reference. Looking forward to see this on print.

(Remarks on code availability)

Code is now available. The Zenodo link is also working.  
I'm sorry, but I don't have the time to review the code.

Referee #2

(Remarks to the Author)

The authors have adequately addressed my comments.

(Remarks on code availability)

Referee #3

(Remarks to the Author)

With the substantial updates and refinements, Wirbel, Hickey et al. have addressed all my concerns and curiosities. Their code and data are now publicly available. I have no further doubt that their findings are both novel and important. I congratulate them on their work.

(Remarks on code availability)

Extremely well-documented code and data. This will be an important resource for the field.

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Referee #1 (Remarks to the Author):

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In short, this manuscript demonstrates the interest of using long-read sequencing for phage metagenomics. It also provides some exciting novel data on the dynamics of prophages and their induction in the gut. Finally, it extends our knowledge on temperate phage host range and discovers a novel type of transposase-driven integrative temperate phage. The manuscript is clear and well-written. I believe this may become a landmark study in the field.

We thank the reviewer for their complimentary and detailed comments on the manuscript. We are pleased that the reviewer feels that this manuscript “may become a landmark study in the field” and that they believe that our findings may have strong implications to the future study of phages in metagenomic samples.

We also appreciate their insightful comments and suggestions (below) that have allowed us to improve the clarity of presentation for phages found in multi-family hosts and the rigor of our analysis of phage dynamics. We believe that addressing the reviewer’s comments in our revised manuscript has greatly enhanced the clarity and impact of our findings.

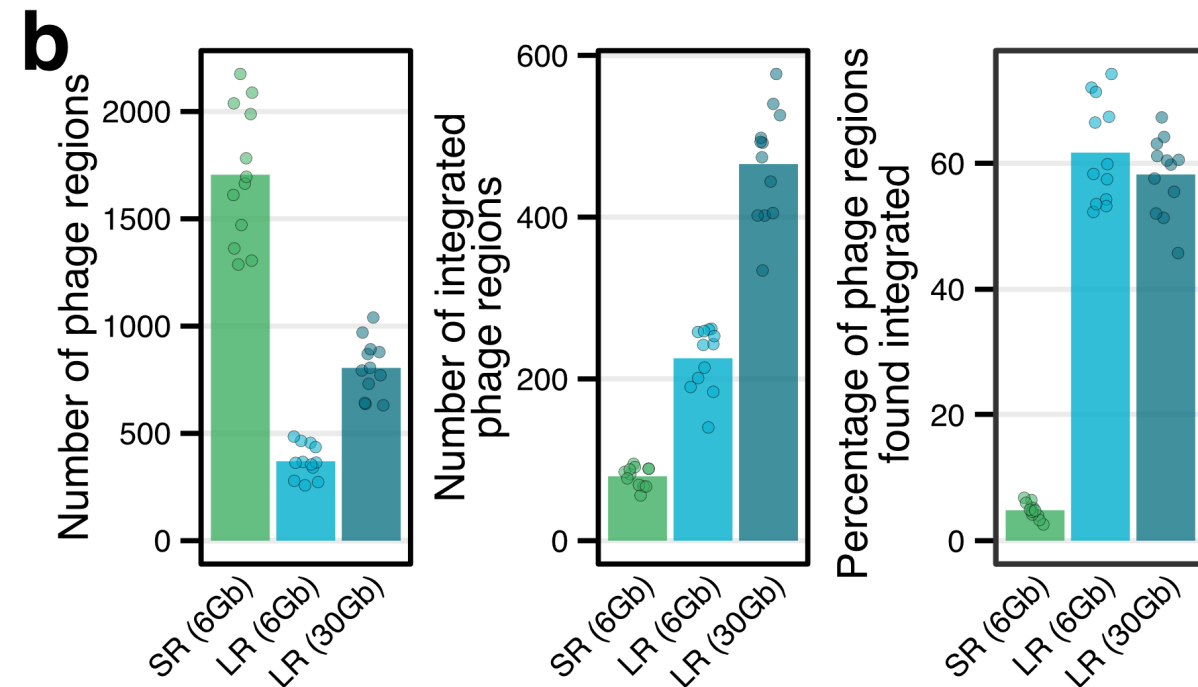
Some comments/suggestions.

- It would be pertinent to comment figure 1b in relation to the relatively low fraction of integrated regions. 60% seems well below the frequency of integrative prophages among prophages. A discussion on how many of the remaining 40% are not integrative prophages and how many are poorly covered integrative prophages would be useful.

We thank the reviewer for the comment, which suggests that we clarify the results in Fig 1b. Addressing this comment, the fraction represented in Fig 1b referred to the proportion of integrated phage regions out of all phage regions. We find that of all phage regions predicted by geNomad on the short read (SR) assembly, only ~5% are predicted to be integrated, whereas for both of the long read assemblies (LR 6Gb, LR 30Gb) the percentage of phages annotated to be integrated is ~60% of all phage regions. This more accurately represents the conclusions from other studies claiming that a majority of phages in the gut are integrated prophages. The remaining 40% are likely a mix of poorly covered fragmented phage genomes (which cannot be assembled into their host context) as well as linear and circular non-integrated phages. Long reads do allow us to recover fully circular phage genomes, which could represent fully episomal DNA that could be obligately lytic phages or phage plasmids.

*Actions taken:*

*We have modified the axis title of Fig 1b to “Percentage of phage regions found integrated” to improve clarity.*



*(revised Figure 1b)*

- Line 206. One needs an histogram of the number of prophages per cluster (to see how balanced it is this distribution) in supplementary material to better understand this result. The other (more important) reason for this plot is related to the statements in line 216 where is indicated that 5% of the clusters were gained or lost. It was not completely clear to me if this concerns the number of phages or clusters (and whether that mattered).

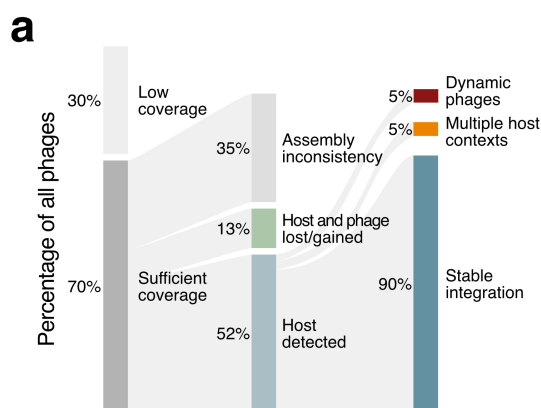
We thank the reviewer for their careful attention to detail. We agree that providing more information about the number of prophages per cluster as well as how many phages are dynamically gained or lost will improve the understanding of this result and how many phages are impacted.

In the result section, we generally refer to the number of phage clusters. However, all dynamic phages will be represented by singleton clusters, as dynamic phages are only detected in a single timepoint. We appreciate that our language was imprecise when we talked about the number of phages versus the number of phage clusters; to remedy this, we have amended the results section.

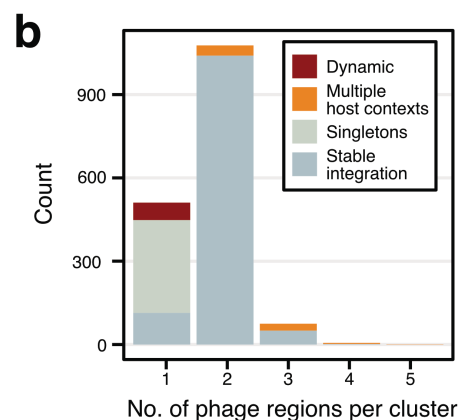
In response to this and other reviewer's comments, we have also amended Figure 2a to more clearly indicate the reference population for the fraction of dynamic phages. Additionally, we have included further filtering steps to remove low-coverage and poorly assembled phage clusters (see response below as well).

#### *Actions taken:*

*We have included the suggested histogram of the number of prophages per cluster used for stability analysis in SFig 5. Additionally, we have adjusted Figure 2a to better reflect our amended analysis approach.*



*(revised Figure 2a)*



*(revised Supplementary Figure 5b)*

- It would be relevant to discuss if these prophages could have been present in the other time point at low frequency and therefore been missed. Some statistics would help too: Are these prophages among the rarest or the most frequent (when present?). And are their hosts changing a lot in frequency? This is a very interesting result that requires some further details.

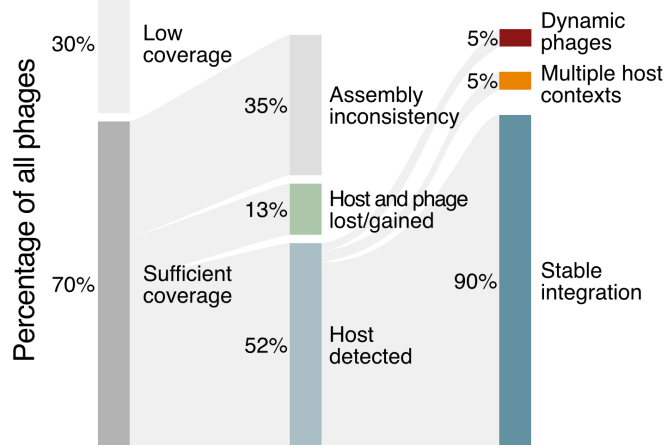
We thank the reviewer for this important comment, which prompted us to investigate our finding of prophage dynamics more deeply and led to a change in the revised manuscript. Indeed, we fully acknowledge that our analysis is based on assemblies, and therefore potentially misses phages that did not assemble due to low abundance.

To address the reviewer's comment, we first quantified the median read coverage for all phages in both timepoints and compared read coverage at each timepoint with our classification of stable or dynamic phages. We discovered that our assembly-based analysis pipeline to cluster and classify prophages together with their host regions resulted in unexpected results: Some "dynamic" phages had high read coverage in both timepoints. We found that this was due to differences in phage annotation, not due to "true absence" of the prophage in question. In our previous classification pipeline, these cases were erroneously classified as dynamic. In some other cases, low read coverage in both timepoints resulted in the assembly of two phages that were highly similar, but just below our identity cutoff to cluster them together as stable phages. Given these unexpected results uncovered in response to this comment, we carefully modified our analysis pipeline, adding the following steps: i) we excluded all phages with low median coverage ( $<10\times$ ) in both timepoints as potentially unreliable, ii) we accounted for potentially disparate phage annotation across timepoints by checking the mapping of phages to the other timepoint's assembly, and iii) we did not count phages at the edges of contigs, since those often resulted in false positive identification of dynamic phages. As an additional quality control, we manually inspected the assemblies for all ( $n = 63$ ) remaining dynamic phages, confirming their assembly into different host contexts. The resulting set of dynamic phages consists of two groups; phages that are lost/gained over time and have no read coverage in the other timepoint and phages that are assembled into different host contexts across timepoints. To clarify these results, we added the median phage read coverage across timepoints to the revised Supplementary Figure 5.

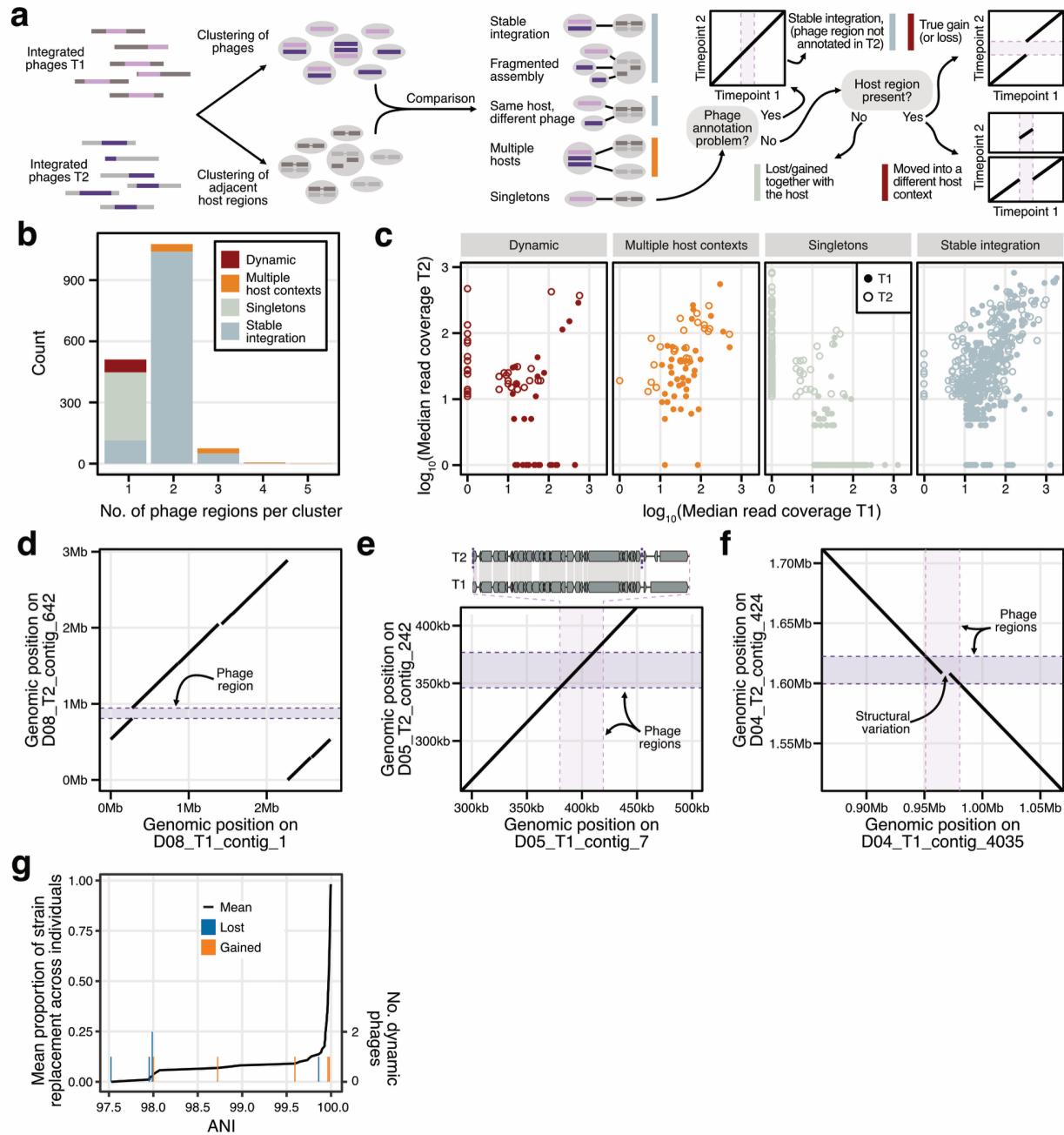
*Actions taken:*

*We comprehensively changed our approach for classifying phage clusters as dynamic or stably integrated, adding coverage filters and additional quality insurance steps. We modified Figure 2a and Supplementary Figure 5 to indicate the updated classification pipeline. Similarly, we changed the main text on page 6, lines 208-218, and the Methods sections on page 19.*

**a**



*(revised Figure 2a)*



(revised Supplementary Figure 5)

- A potential confounding variable is the detection of the strain. One needs more details on what was done to ensure that the strains at T1 and T2 are the same. If in a certain percentage of cases they are closely related strains, then this would contribute to explain the 5% of gained or lost prophages.

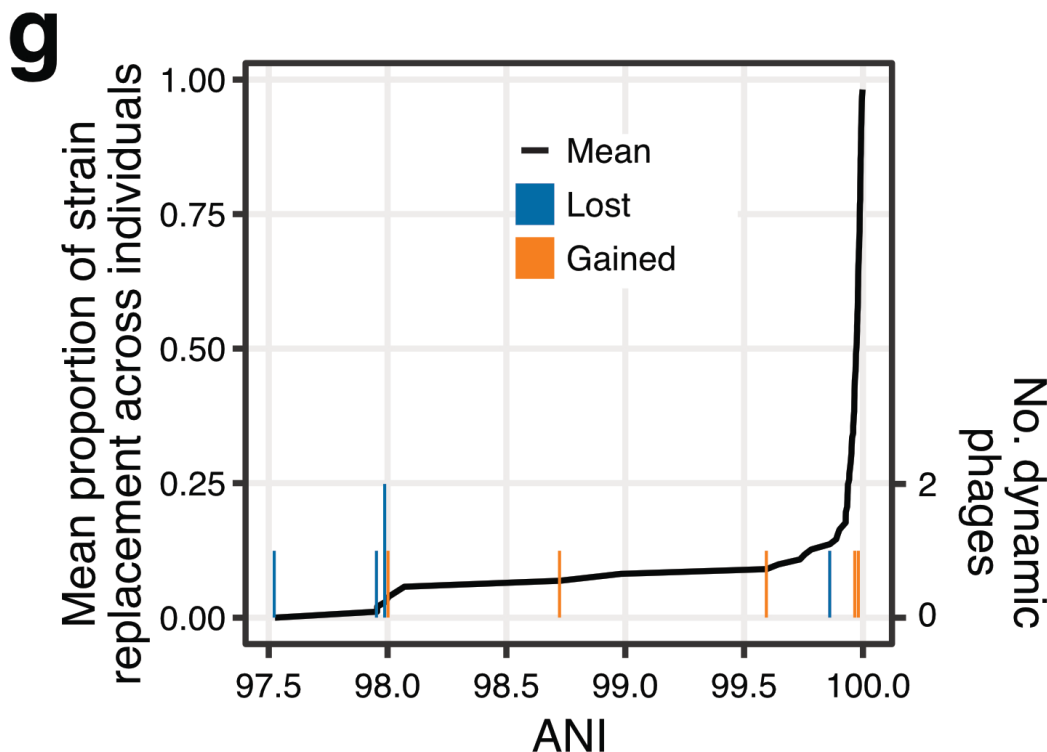
We thank the reviewer for their thoughtful comment. We understand that this comment aims to clarify whether there is bacterial strain replacement occurring between the two timepoints, which could be explaining some of the gained or lost prophages. We acknowledge that strain



replacement is a process that does contribute to the detection of ‘dynamic’ phages. For example, by closely investigating a few examples, we uncovered evidence of strain replacement at the bacterial level (see for example Supplementary Figure 7).

To address this question and evaluate this phenomenon at a larger scale, we decided to compare high quality, high coverage (median coverage > 30) MAGs shared between timepoints of the same individual and compare nucleotide identity (using FastANI). Using this approach, we identified ~15 high quality, high coverage (>30x) MAGs that are shared, per individual. By selecting only high quality MAGs with high coverage we tried to minimize assembly errors that typically occur in low coverage MAGs as well as those with high contamination or low completeness.

With this set of MAG pairs, we could calculate the rate of strain replacement for different ANI cutoffs (see Figure below). With a relatively relaxed cutoff below 99.5% ANI, we detect ~9% strain replacement; however the true cutoff for strain identity likely falls in the sharper part of the curve between 99.9 and 100% ANI.



(revised SFig. 5g)

To evaluate whether strain replacement explains detection of dynamic phages, we plotted the number of dynamic phages (lost or gained) from paired MAGs against the paired FastANI value. While the number of dynamic phages detected in this refined dataset is small ( $n = 10$ ), we see that some (6 of the 10) dynamic phages are likely the result of strain replacement (<99.0 ANI). Four of the six of these events are due to the phage being lost from the population (red vertical lines in the graph). This fits with the logic that most prophage excision events are the result of

phage replication and subsequent lysis and death of the host. We are also able to detect the gain of two prophages within high similarity MAGs (>99.9 ANI; the two blue vertical lines on the far right of the graph) which are likely to represent infection events. While it is hard to extrapolate from this analysis, we believe that we are able to detect the prophage dynamics of both strain replacement and integration/excision events.

*Actions taken:*

*We have included this finding in Supplementary figure 5 and modified the relevant result (page 6, line 220-224) and discussion (page 14, line 476-479) sections to emphasize the contribution of strain replacement to our detection of dynamic phages.*

- If there is an heterogeneity in the presence of prophages in a population, some of the 5% of gains/losses actually result from variants that never got infected by the prophage. This interaction between the two factors (non-ubiquity at a time point and gain/loss between time points) needs to be quantified.

We thank the reviewer for their comment and agree that our quantification of dynamic phages could result from strain replacement of non-infected lysogens. In light of the comments regarding the classification of stable and dynamic phages, we have changed our approach to improve the accuracy of the detection of dynamic phages, as described in the response to other comments above. In response to this and other comments, we have also found that only 37% of the dynamic phages overlap structural variation (SV) calls from Sniffles. We would have expected most dynamic phages to be represented in the set of SVs detected by Sniffles, however the remaining dynamic phages often overlap SVs that are filtered out by Sniffles. This highlights a slight bias towards specificity over sensitivity in our analysis. We investigated the subset of dynamic phages with SV overlaps ( $n = 23$ ) to investigate whether there was non-ubiquity at the timepoint in which they were found. Of those 23, 20 have evidence of reads supporting a deletion, despite the assembly of the phage region. Therefore, a majority of dynamic phages do have evidence of heterogeneity at the original timepoint. This supports that some 'loss' events could be the result of selection on standing variation or that 'gain' events could be explained by infection of only a subset of all potential host cells by a given phage.

*Actions taken:*

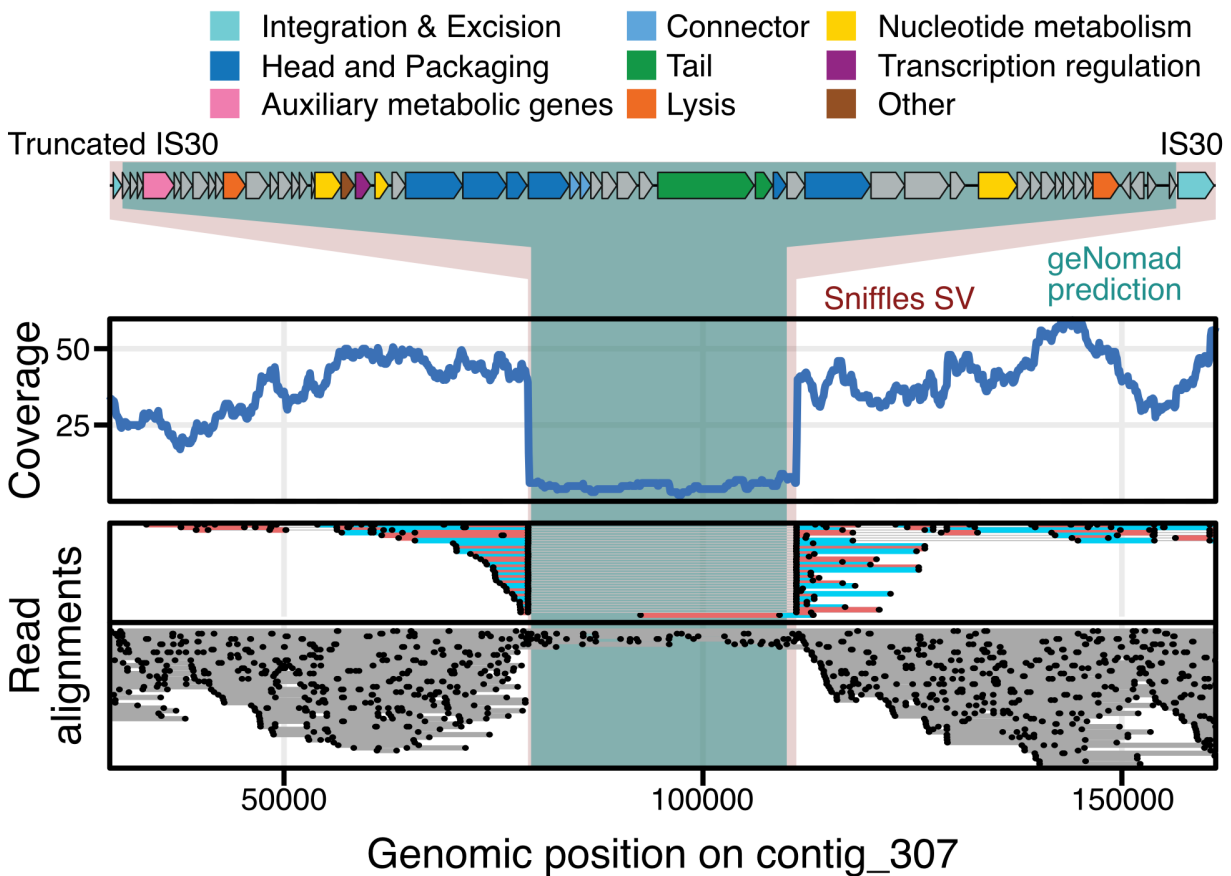
*We have modified the results on page 8, line 259-261.*

- Insertions and deletions are measured by using the average coverage. While this is sometimes rather obvious (fig 2b), the data on heterogeneity in the population is less trivial. For example, in figure 2d there is a lot of variance outside the prophage region (and I assume other cases are even more subtle). As a result, one needs more substantial statistical evidence that the existence of coverage in the prophage region is not the result of such variations. If the page was completely absent, then no reads should have been obtained, but given the variance one needs some information on the intervals of confidence for the % of presence of the prophage. In the same vein, the comparisons between the sniffles analysis and Genomad, seems to assume the previous is absolutely correct, which needs characterisation/justification.

We thank the reviewer for their comment and would like to clarify our approach. In fact, insertions and deletions of phages are actually not measured by average coverage. Instead, insertions and deletion of phages were quantified using Sniffles. We used the coverage of selected phage regions only to better illustrate phage dynamics, not for identification. Therefore, we are able to detect prophage insertion/deletion (and duplications) also in cases with variation in coverage, since the clustering of supplementary alignments is the basis for structural variation detection (instead of coverage variation). We apologize for the lack of clarity in our original submission and, in response to this comment, have revised the main results text to better reflect our analytical approach, which is in keeping with the reviewer's preferred method of calling SVs.

A second concern raised by the reviewer's comment relates to us treating the phage boundaries identified by the Sniffles analysis as the gold standard for the comparisons against phage predictions from geNomad (and other phage identification tools). It is true that SVs identified by Sniffles calls are not always correct, especially given the complexity of some variable regions or mis-assemblies. However, we do believe that our set of phage-SV overlaps represent the true boundaries of integrated prophages. We manually inspected many of the Sniffles calls overlapping phage regions (see for example SFig 6) to further increase our confidence in the Sniffles SV calls. For the following reasons we believe that it is valid to treat the SVs identified by Sniffles as the gold standard:

1. The SV identification by Sniffles is rather conservative and specific (in contrast to being overly sensitive), as only ~37% of dynamic phages overlap SVs identified by Sniffles. For the remaining dynamic phages (which should result in detectable SVs), Sniffles often detects SVs, but filters them out, mostly because of relatively low read coverage. We therefore believe that the majority of SVs that remain after those filters represent genuine SVs.
2. Phage prediction tools are based on predicted protein sequences, which can be erroneous and therefore impact the exact prophage boundaries. By contrast, SV detection is based on direct read evidence and therefore not subject to possible gene annotation errors. As an example, the phages shown in SFig. 5e represent the same, stably integrated phage, but they did not cluster together since the phage predictions did not overlap sufficiently. The reason for this discrepancy is a single nucleotide difference resulting in a differently predicted protein, thereby changing the boundary of the phage prediction. We therefore are more critical of the exact boundaries from phage predictions, as those are more sensitive to single nucleotide assembly errors.
3. Another reason why we deem the boundaries identified by SVs as gold standard over the phage predictions is the gene content of the final phage genome. We would like to illustrate our reasoning with an example from the IScream phages: In this example, the IS30 elements on either side of the phage genome are not included in the phage prediction from geNomad (see Figure below). However, the read evidence clearly shows the exact boundaries of the integrated prophage. As the IS30 elements are a conserved feature of these types of phage genomes and a potential explanation for their mobilization machinery (as the genome is lacking other integrases), we believe that the phage genome as identified through Sniffles SV more realistically reflects the true phage genome.



*Actions taken:*

*We have revised the result section to increase the clarity of our presentation, adjusting page 7, lines 241-247.*

- Line 469. Interesting hypothesis, but it was not clear to me how this could work. I agree that induction requires functional prophages. But since there is super-infection exclusion these phages would go nowhere if the bacteria in the vicinity are lysogens even if partially defective (except if defect concerns specifically the exclusion mechanisms). This could be some sort of bet hedging, where a small population excises to “explore” the possibility that some sensitive cells are present in the population where the majority remains in the existing favourable host. This would fit the data showing that lysogens and non-lysogens co-exist.

We thank the reviewer for their consideration of our hypothesis and the interesting suggestion of an alternative hypothesis. We agree that induction without susceptible hosts nearby would be fruitless, and find the exploratory bet-hedging idea compelling and a great addition to the discussion. Based on our analysis, it seems that about 7% of the detected lysogens have co-existing uninfected hosts. This may support the model proposed in Shkoporov et al. where mechanisms such as phase variation result in a small susceptible sub-population allowing for long-term phage persistence (see PMID: 34407825). Our original hypothesis stemmed from this model where there may be a constant supply of susceptible hosts and alongside low-level

induction, this could ensure propagation of functional prophages in species that have long-term persistence with their host.

*Actions taken:*

*We have revised lines 492-495 on page 14 to incorporate the suggested hypothesis and clarify our original suggestion.*

- I had some difficulties with Figure 3. Occasionally, phage regions (green rectangles) are smaller than the conserved region. I guess this is Genomad getting edges wrong. But in panel d, one wonders what is the real structure in terms of what is between the two rectangles (each is too small to be a prophage) and what is the third region of similarity (a satellite?). It doesn't fit the narrative about integration/excision that coverage does not match the prophage prediction (which is smaller) neither the homologous region (which is larger). I also find surprising that the regions around the prophage are always non-homologous. This seems more expected in panels d and e (different species), but not in a. This example would be more clear if one could have in supplementary material a figure with gene names/functions.

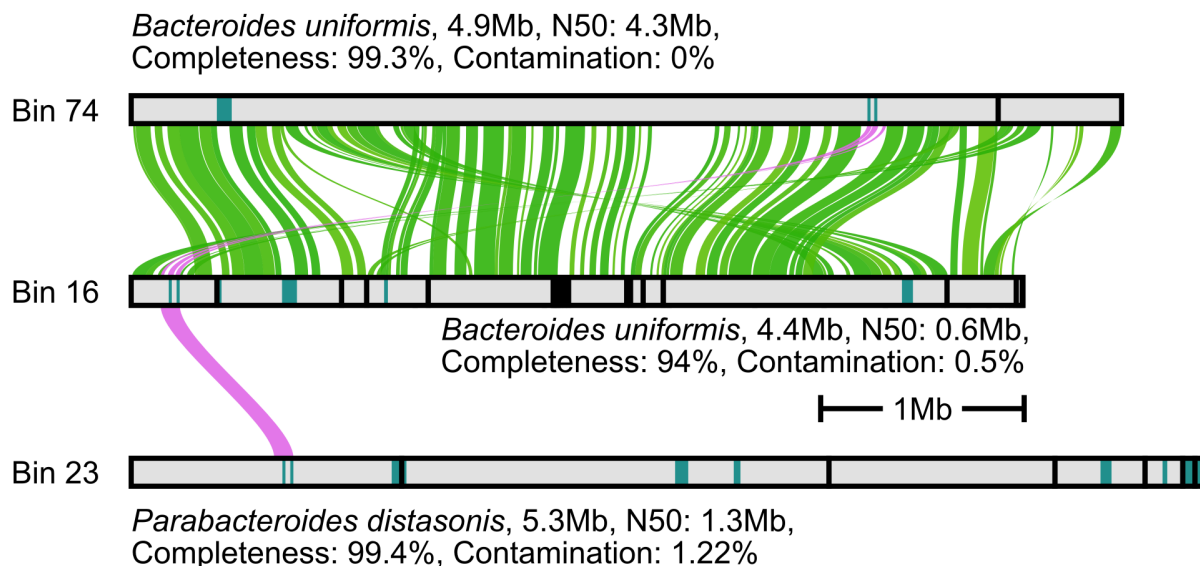
We thank the reviewer for their detailed comments on and suggestions for how to improve Figure 3. In response to this and the other reviewer's comments, we have extensively re-designed Figure 3 and the accompanying Supplementary Figures to improve the clarity of our presentation. We would like to clarify some of the questions raised by the reviewer, which we addressed in our revised manuscript.

**1. Phage region versus conserved region:** The reviewer is correct in their assumption that the geNomad predictions are not always perfect and slightly over- or under-estimate the size of the region of homology (see also our response to the comment regarding the comparison between geNomad and Sniffles). To improve clarity, we only indicate the region of homology between genomes in the revised Figure 3. However, each of the regions overlaps with a region predicted to be a phage by geNomad (new Figure 3b and 3d). The example in question (much larger region of homology, original Figure 3e) has been moved to the supplement. In this case, the phage prediction from geNomad was relatively short (~10kb), while the actual region of homology was about 35kb.

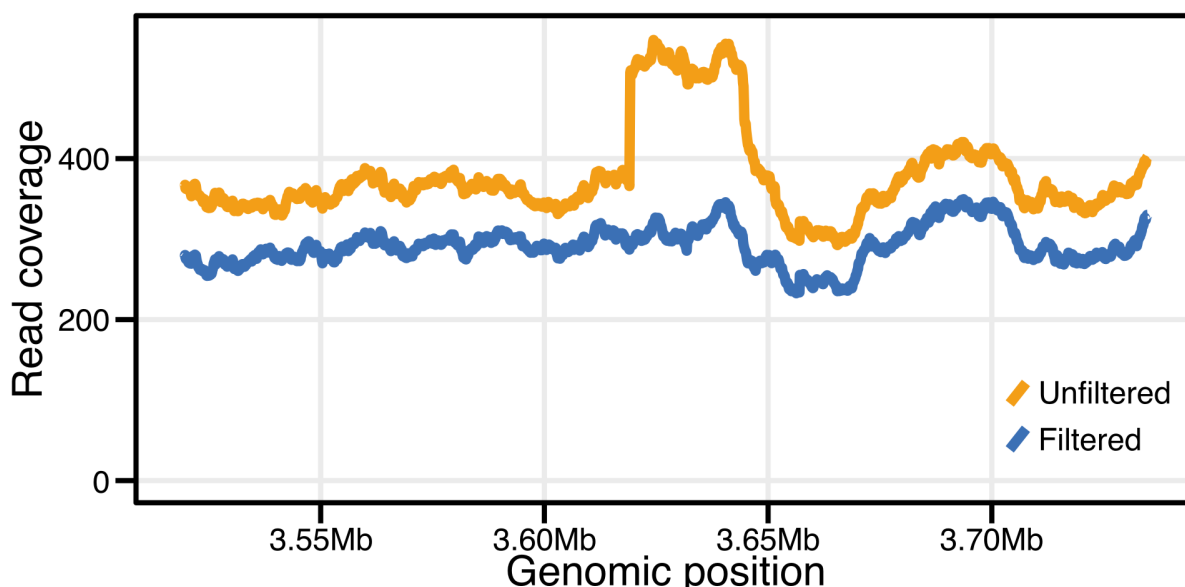
**2. Multi-region *Bacteroides* example:** The reviewer raises a very interesting point. Indeed, in the example shown in the original Figure 3d, there appear to be 3 regions of homology, two of which are also predicted to be phages by geNomad. Upon closer inspection of the cluster in question, we consolidated two of our eight clusters with phages integrated into different bacterial families, represented by the two geNomad predictions (present in multiple genomes of the *Bacteroides* and *Parabacteroides* genera). As those regions are supported by high coverage, a lack of read end pile-up, and taxonomic annotations of surrounding genes (see also comment to the other reviewer), we do believe that these regions represent genuine horizontal gene transfer between taxonomic families. However, we are only able to speculate about whether this region represents a phage genome, another type of mobile genetic element or a region of homologous recombination independent of a gene transfer enzyme (such as recombinase). Focusing on three bins in which the complete homologous region was assembled into a broader genomic context

(see Figure below), we found the homologous region to be fully and syntenically shared between two different bins representing (i) *Bacteroides stercoris* (bin 16) and (ii) *Parabacteroides distasonis* (bin 23); (pink ribbon connecting bins 16 and 23, below). The example shown in the original Figure 3 involved the same *P. distasonis* assembly, but a different *B. stercoris* assembly (bin 74, assembled from another donor). Indeed, the two *B. stercoris* assemblies represent different strains of the same species (97.6% ANI), which have evidence of extensive genomic reorganization with respect to one another (green ribbons between bins 74 and 16; also in the phage region in question, highlighted in pink ribbons between bins 74 and 16). Potentially, the region in question represents a more ancient horizontally acquired region that is involved in homologous recombination events within *B. stercoris*.

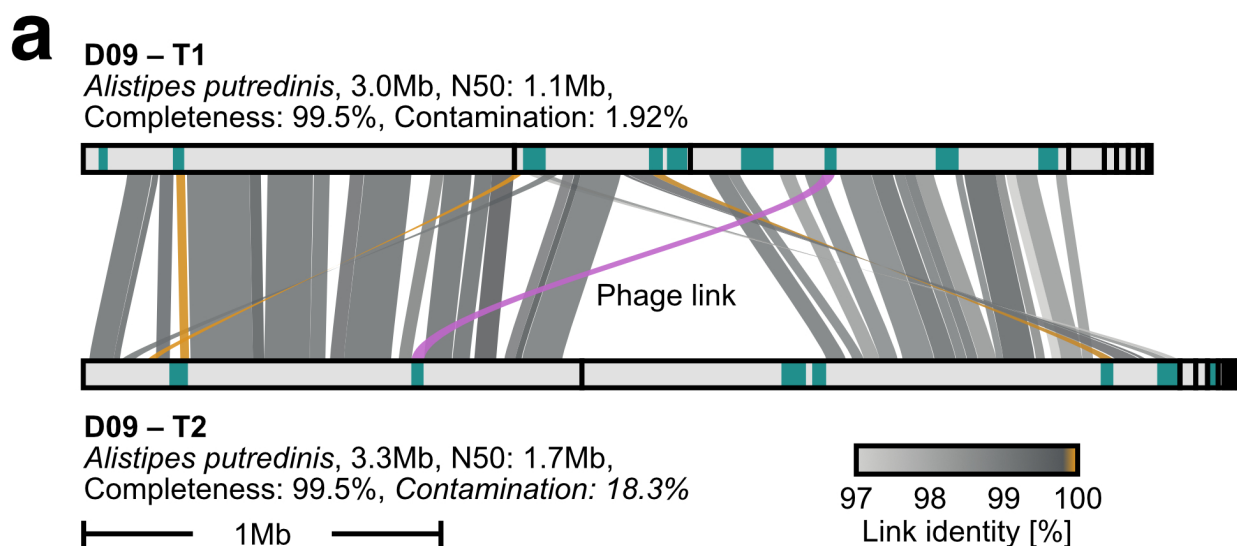
Given the complexity of this specific example, we moved it to the supplement and added a small discussion in the figure legend.



**3. Coverage increase:** We thank the reviewer for spotting this unexpected finding which prompted us to investigate it in more detail. The reviewer is correct in that this higher coverage does not fully overlap with the putative phage region or region of homology. Thus, this calls for an alternative explanation. To determine what caused this unexpected finding, we manually inspected the coverage pile-up using IGV. We observed that the higher coverage was mostly due to a subset of reads that mapped with a large fraction of mismatches in their alignments, consistent across these reads. This would appear to be mismapping. Indeed, when we filter out reads with more than 1% of mismatches in their alignments, the previously observed higher coverage for this region is absent (see Figure below). The reason for these mismappings could be that the region in question is present in two bacterial species within the same sample, leading to supplementary alignments, which artificially inflates coverage. As a result, we included an additional filter for the fraction of mismatches when computing coverage for the multi-host phages.



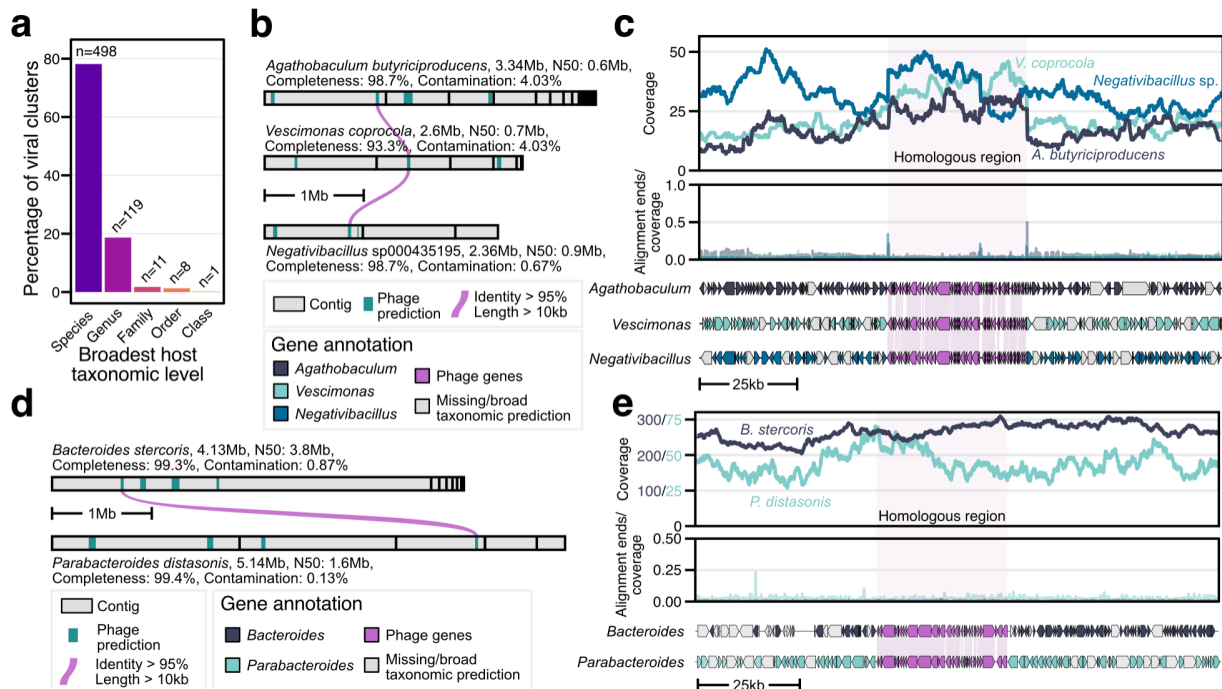
**4. Alistipes example:** The example of original Figure 3a was a phage that we had found to be integrated into *Alistipes putredinis* in both timepoints. However, the surrounding host regions were not the same across timepoints, therefore resulting in the classification of this phage as a 'multi-host' phage. Looking more closely at this example, we found that the two *Alistipes* bins exhibited large structural variations between timepoints and limited nucleotide similarity, indicating that two different strains of the same species were present across timepoints. If the same strain had persisted over time, we would have expected highly similar assemblies in both timepoints. We appreciate that the zoomed-in comparison in Figure 3a failed to convey the broader context of the similarity (and differences) across bins and have since moved this analysis to a re-designed supplementary figure, showing a broader comparison across bins. A comparison of the two different *A. putredinis* bins is shown below, with the phage region highlighted in pink.



*Actions taken:*

*We have extensively re-designed Figure 3 and Supplementary Figures 7 & 8. Additionally we adjusted the results on page 9, line 299-326 and the Methods on page 20, line 742-753.*





#### Minor comments.

- Line 55. I would suggest to introduce Cre differently, because implicitly the text suggests it's an integrase of an integrative phage, when it's a resolvase of P1 (as stated later in the text).

We agree with the reviewer that the justification for the proposed IScream phage name lacked consistency, as Cre in P1 is a) a tyrosine recombinase, not a DDE recombinase, and b) Cre is not used for integration into the host genome.

Internally, we had dubbed our discovery of IS-bounded phages as IScream phages, as the genomic organization of the IS-bounded phages reminded us of an ice cream sandwich (the rest of the phage genome sandwiched between IS elements). In our original submission, we had tried to tie our preferred name to existing phage nomenclature, but we fully appreciate that the post-hoc reasoning was implausible. In response to this comment, we have adjusted the section in question to indicate the original reason for the name.

#### Actions taken:

We adjusted the text on page 11, lines 369-370 to rectify the reasoning for the naming of the IS-bounded phages.

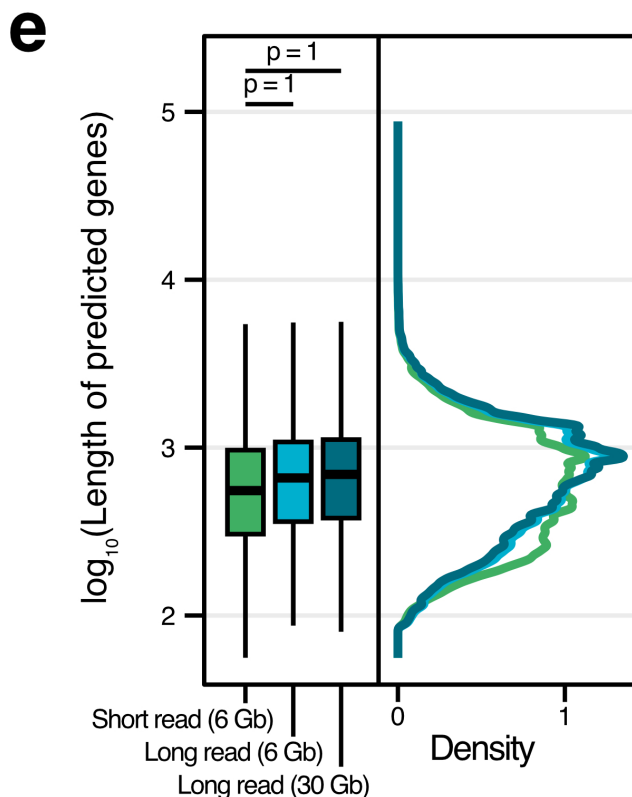
- Figure S1, panel E. The authors should check the underlying data. The three box plots seem strictly identical, which seems really unlikely. There may have been a problem in plotting the data. We appreciate that the difference between the three distributions is hard to assess visually in SFig. 1e. To clarify, we provide here the values used for plotting the box plots:

|  | Lower<br>whisker | 25th<br>percentile | Median | 75th<br>percentile | Upper<br>whisker |
|--|------------------|--------------------|--------|--------------------|------------------|
|  |                  |                    |        |                    |                  |



|                      |    |     |     |      |      |
|----------------------|----|-----|-----|------|------|
| Short read           | 56 | 305 | 554 | 965  | 1544 |
| Subsampled Long read | 62 | 362 | 659 | 1079 | 1734 |
| Long read            | 61 | 380 | 692 | 1115 | 1794 |

As the small differences in absolute values are extremely hard to appreciate on the logarithmic scale used for plotting, we additionally explored an alternative visualization to more easily compare the distributions across sequencing technologies. The revised visualization, combining boxplots and density plots next to each other, is included below and in the revised manuscript.



*Actions taken:*

*We adjusted the visualization in SFig. 1e to better show the differences between gene length distributions.*

- Line 284 and discussion. Beyond metagenomic studies, the existence of phages that were experimentally shown to transfer across genus had been known for a while (PR5, P1 or PRD1). The quantification here done is interesting, nevertheless.

We appreciate the reviewer's perspective on the framing of our results and apologize for the oversight of omitting this relevant information. We adjusted the result section and the discussion to clarify that phages with host ranges spanning different bacterial genera had been demonstrated previously with molecular studies.

*Actions taken:*

*We adjusted the results section (page 8, lines 283-287) and the discussion (page 15, line 498) to indicate that phages with broad host range (spanning different bacterial genera) have been characterized with molecular studies.*

- While I appreciate the name ISCream phages I found the use of Cre in the name misleading, since Cre is a tyrosine recombinase.

We thank the reviewer for their perspective on the introduction of the IScream phage name. Internally, we had dubbed our discovery of IS-bounded phages as IScream phages, as the genomic organization of the IS-bounded phages reminded us of an ice cream sandwich (the rest of the phage genome sandwiched between IS elements). In our original submission, we had tried to tie our preferred name to existing phage nomenclature, but we fully appreciate that the post-hoc reasoning was implausible. In response to this comment, we have adjusted the section in question to indicate the original reason for the name.

*Actions taken:*

*We adjusted the text on page 11, lines 369-370 to rectify the reasoning for the naming of the IS-bounded phages.*

- Line 472. Not clear to me which data suggests that phages have evolved to be more specific and narrow host range (suggesting this is a macro-evolutionary trend). I'm afraid I don't get that from the cited paper. The precise meaning of the statement should be clarified.

We thank the reviewer for their thorough examination of our discussion and for bringing this point to our attention. Upon careful re-reading of the cited work, we agree that this original statement was not an accurate reflection of the conclusions regarding the host range of phages. We have amended this sentence to more accurately reflect our meaning.

*Actions taken:*

*We adjusted page 15, line 497.*

- The Lopez et al preprint is now published.

We adjusted the reference in the revised manuscript.

- Some references lack page numbers or ids: 30, 35, 71 or have irrelevant text : 15

We thank the reviewer for their thoughtful attention to detail and apologize for our oversight in missing these incomplete references. We adjusted the references in question in the revised manuscript, except for the publication from Cook and colleagues, as this reference does not have a page number as per the official citation from the journal.

Referee #2 (Remarks to the Author):

Wirbel et al., in “Discovering...”, present a study of phages in the gut microbiome using long read sequencing. Twelve samples were studied from six healthy individuals studied over two years. The authors find that phage populations are generally stable over time. Sometimes host bacteria exist as mixed populations where some but not all members of the species contain specific integrated prophages. A few phages were found to infect multiple hosts. A lineage of phages (IScream) was identified that was proposed to use IS30 machinery for integration. Follow up studies suggested that the IScream sequences appeared in virus-like particles released from cells.

The long read sequencing method is useful for documenting phage genomes comprehensively. Some of the points on phage dynamics have been reported previously or anticipated in other work, such as longitudinal stability and low levels of induction in gut. Still, this study presents a useful overview with improving technology, and adds some interesting points, such as observing bacterial strains with and without specific prophage, and the finding that a few human gut phages seem to have broad host ranges.

The demonstration of naturally occurring phages that use IS30 machinery for site specific integration is novel and interesting. Previously phage Mu was shown to use a DDE-type enzyme for transposition to allow genome multiplication prior to packaging; IS30 transposase is also predicted to be a DDE-type enzyme. IS30 was also shown to be able to substitute for lambda integrase to support bacteriophage replication (ref 41). Still, it is quite interesting to see the apparent wide use of this mechanism documented in metagenomic data.

We thank the reviewer for their thorough reading of the manuscript and their valuable comments. We appreciate their insight as to how this work fits into the ongoing discussions about the dynamics of gut phages. The suggested edits and additional experiments significantly improved the manuscript and we are grateful for the opportunity to include these revisions.

Minor points.

In the Discussion the authors write: “Previous studies investigating human gut virome dynamics, based on short-read VLP sequencing during a period of up to a year or using bulk metagenomic sequencing over shorter time frames (10 days), have reported generally high stability and individuality of the virome<sup>20,27,48</sup>.” Actually reference 20 describes sampling over 2.5 years analyzed by metagenomic sequencing.

We thank the reviewer for alerting us to our oversight regarding the timeframe of the publication by Minot and colleagues.

*Actions taken:*

*We adjusted the sentence in question and changed it to: “Previous studies investigating human gut virome dynamics, which used short-read VLP sequencing (ref Minot et al., Shkoporov et al.) or bulk metagenomic sequencing over shorter time frames (10 days, ref Chen et al.), have reported generally high stability and individuality of the virome. Using bulk, long-read metagenomic sequencing and a longer sampling period of two years, we observed most prophages to be stably integrated in our dataset, consistent with stability estimates for the virome”.*

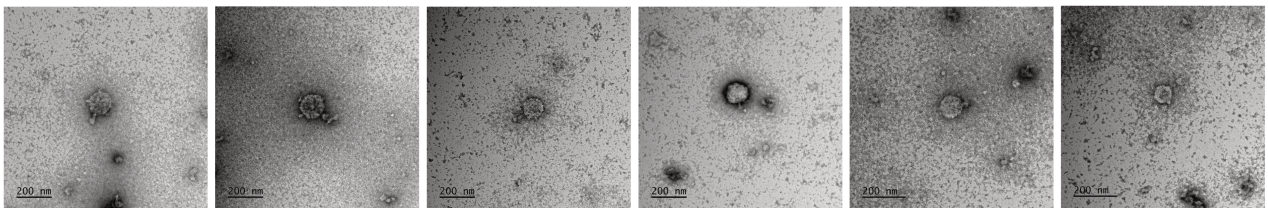
Given that it is possible to grow an IS30-using phage, maybe it would be nice to include a particle EM image?

We would like to thank the reviewer for their suggestion: we fully agree that a particle EM image would be a nice addition to our analysis of IS30-bound phages. We have taken transmission-electron microscopy (TEM) images of the lysate from our *B. hansenii* overnight cultures and identified particles that we believe represent phage particles. These images are now included in the revised SFig. 10. Even though *B. hansenii* harbors multiple integrated prophages (see Fig. 4d), we suspect these particles are likely the IScream phage in question, as our long-read sequencing data indicated this phage to be the only one showing evidence of spontaneous induction, as measured by higher coverage of this phage genome than the bacterial chromosomal DNA and evidence of independent, circular phage genomes of the IScream phage, only (SFig. 10).

*Actions taken:*

*We have taken TEM images of lysate from B. hansenii overnight culture in normal lab conditions and identified particles that we believe to be an IScream phage. We have included these images in the revised SFig. 10. We also changed the results (page 12, lines 420-425) and the methods (page 23, lines 872-881).*

**f**



Referee #3 (Remarks to the Author):

Wirbel, Hickey et al, provide a useful look at phages in long read gut metagenome assemblies. Using very creative bioinformatics analyses, they've uncovered new biology and provide a strong case that existing virome tools can be improved, especially when it comes to prophage boundary identification.

I found both the data and methodologies to be quite compelling. Soon the metagenomics field will be awash in long read-based metagenomic assemblies, and this manuscript helps lay the groundwork for understanding these data.

We thank the reviewer for their insightful comments and attention to detail. We are glad that the reviewer found the 'data and methodologies to be quite compelling'. We also appreciate that the reviewer saw the value this work could provide to the field as more long-read studies are conducted on metagenomes for the study of viruses and that the aim of our study was clear. We greatly appreciate that the reviewer allowed us to expand the impact of our findings through new computational tools and improved rigor.

Critiques and questions:

The authors state that prophage dynamics are low (~5%). However, since only 35% of bacterial chromosomes can be recovered between samples, perhaps the fraction is more like  $(5\%/35\% = \sim 14\%)$ . Please comment on this.

We would like to thank the reviewer for this comment. We agree that this distinction is important, as not all bacterial hosts are stable across time.

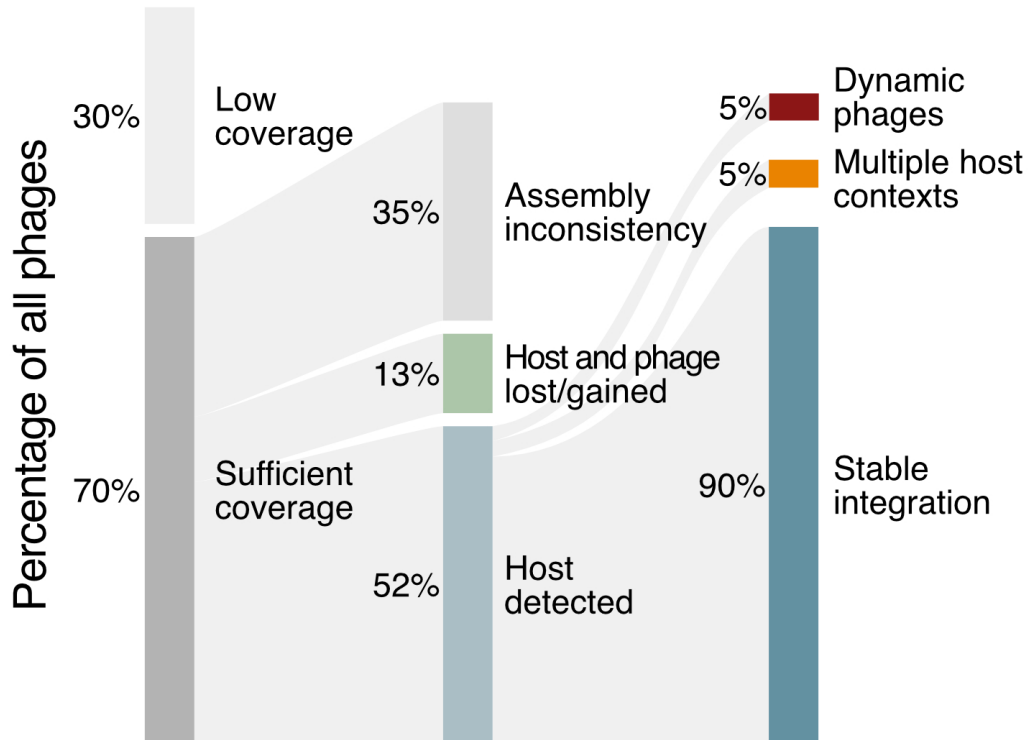
In the revised version of the manuscript, we have modified our pipeline to confidently detect dynamic phages and improve the specificity of our approach. In particular, low-coverage and/or fragmented assemblies make it hard to confidently assess whether the phage region in question was found in the same host context or not. We therefore restricted our analyses to phages with sufficient coverage and confident clustering results. Given these restrictions, we arrive at ~13% of phages not being detected because of dynamics in the bacterial community, whereas the hosts are stable for the remaining 52%. Please note that the overall number of phages used for this comparison is quite different from the original submission, as we tried to emphasize the specificity of our finding by filtering out low-coverage phages. Within the group of phages whose bacterial host contigs were detected through assembly, ~4.7% phages were classified as dynamic. This is equivalent to ~3.8% of phage clusters with confident assemblies (including phages that are lost/gained together with their host) or to ~2.4% of all phages with sufficient coverage (including phage clusters with assembly problems).

In response to this comment, we have re-designed Figure 2a to more clearly convey our analysis approach and to better show the reference population for the fraction mentioned in the text.

*Actions taken:*

*We modified Figure 2a to better represent the reference population for the calculation of the fraction of dynamic phages (see below). Additionally, we changed the main text on page 6, lines 208-218 in accordance with these changes.*

**a**



*(revised Figure 2a)*

Assembly errors still occur in long read-based assemblies. This gives me some skepticism over claims that some prophages are able to integrate in bacteria from other families. For phages mentioned in Fig 3, S7, and S8, can you do due diligence to those assemblies. Ideas include using another assembler (myloasm?) or checking that the phage region read methylation calls match the rest of the (host) contig.

We thank the reviewer for their perspective and fully agree that additional scrutiny needs to be applied to our claim of prophages integrated into bacteria from different taxonomic families. In our original submission, we had tried to provide evidence for this claim by evaluating the coverage of the region in question, as mis-assemblies would potentially be accompanied by dramatic drops in coverage. However, we appreciate that additional, stronger evidence is needed.

To address this point and to increase our confidence in the assemblies, we quantified the number of read ends per genomic window, as mis-assemblies would be apparent by a cluster of read alignments ending in the same position, potentially with a large clipped region (mapping to another contig). This analysis is comparable to the approach applied in the preprint by Trigodet et al., in which the authors identified mis-assemblies by identifying genomic positions where 100% of reads showed alignment ends.

In our revised manuscript, we have adjusted the visualization of the broad host range phages, also in response to comments from other reviewers. Specifically, we have added the number of

read ends divided by read coverage per genomic window as another metric to support the presence of similar phages in taxonomically distant bacterial hosts. Additionally, we added the taxonomic prediction for individual genes from the mmseqs2 taxonomy module, which similarly supports the taxonomic annotations and consistency for the contigs in question.

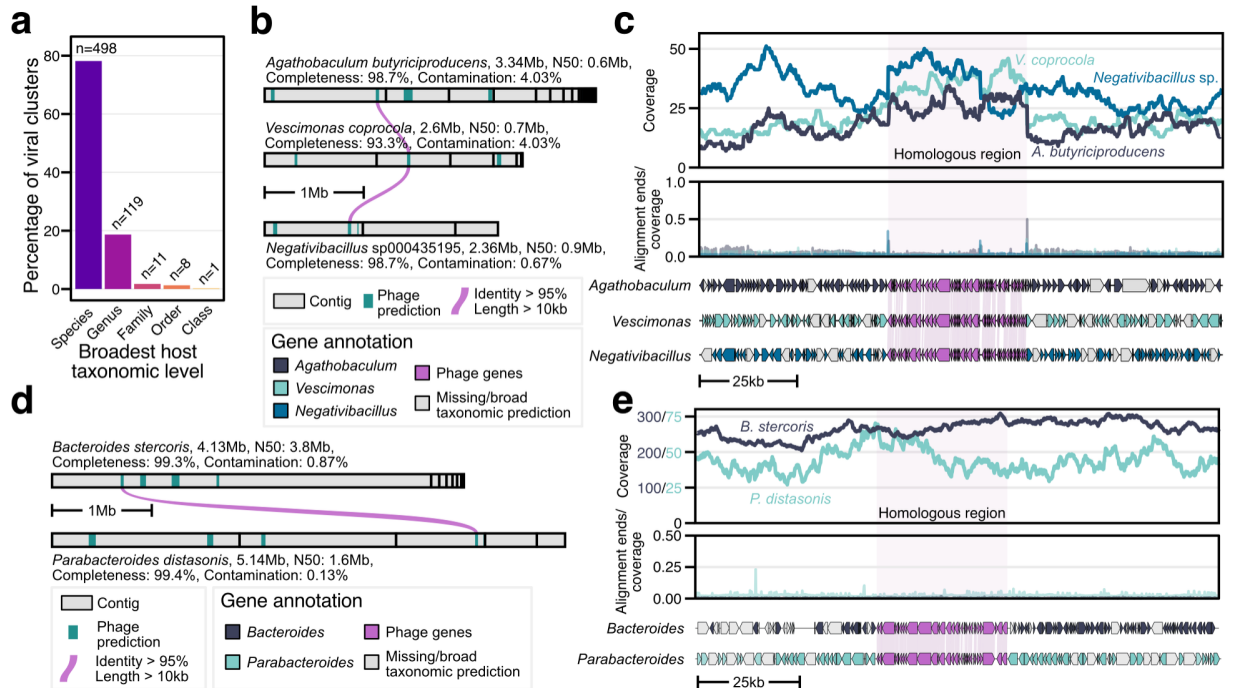
As stated in the original manuscript, the phage cluster encompassing phages in distant bacterial classes is only weakly supported by coverage. In this and in some other clusters, phages are sometimes located at the beginning or end of contigs, which could hint towards potential mis-assemblies. To explore these cases in more detail, we identified the corresponding contigs in the myloasm assembly, as suggested by the reviewer. In two cases, the myloasm assembly was more contiguous than the flye assembly, providing additional support for the integration of these clusters into different bacterial families.

Overall, we believe that our claim of phages integrated into different bacterial families is sufficiently supported by the presented data, since we now provide support through four independent approaches: i) coverage, ii) the absence of the accumulation of read ends, iii) consistent taxonomic annotations of the flanking regions, and iv) orthogonal assemblies through myloasm.

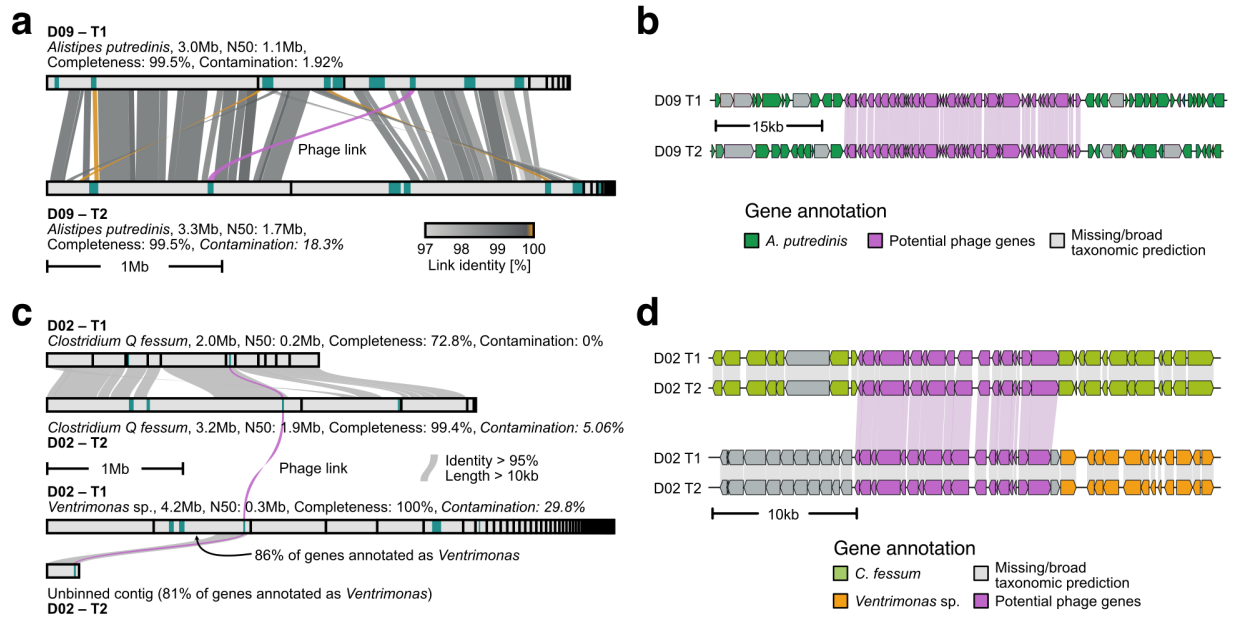
*Actions taken:*

*We significantly revised Figure 3 and Supplementary Figures 7 and 8 to better visualize our confidence in the assemblies by adding the number of read ends (divided by coverage) to each genomic position. Additionally, we visualized the taxonomic predictions from mmseqs-taxonomy for the surrounding genes to show the taxonomic consistency of the region in question. Lastly, as suggested by the reviewer, we applied myloasm as an alternative assembler to our data and investigated the phage clusters at contig ends in more detail (SFig. 8). We modified the corresponding results on page 9, lines 299-326 and the methods on page 20, lines 742-753.*



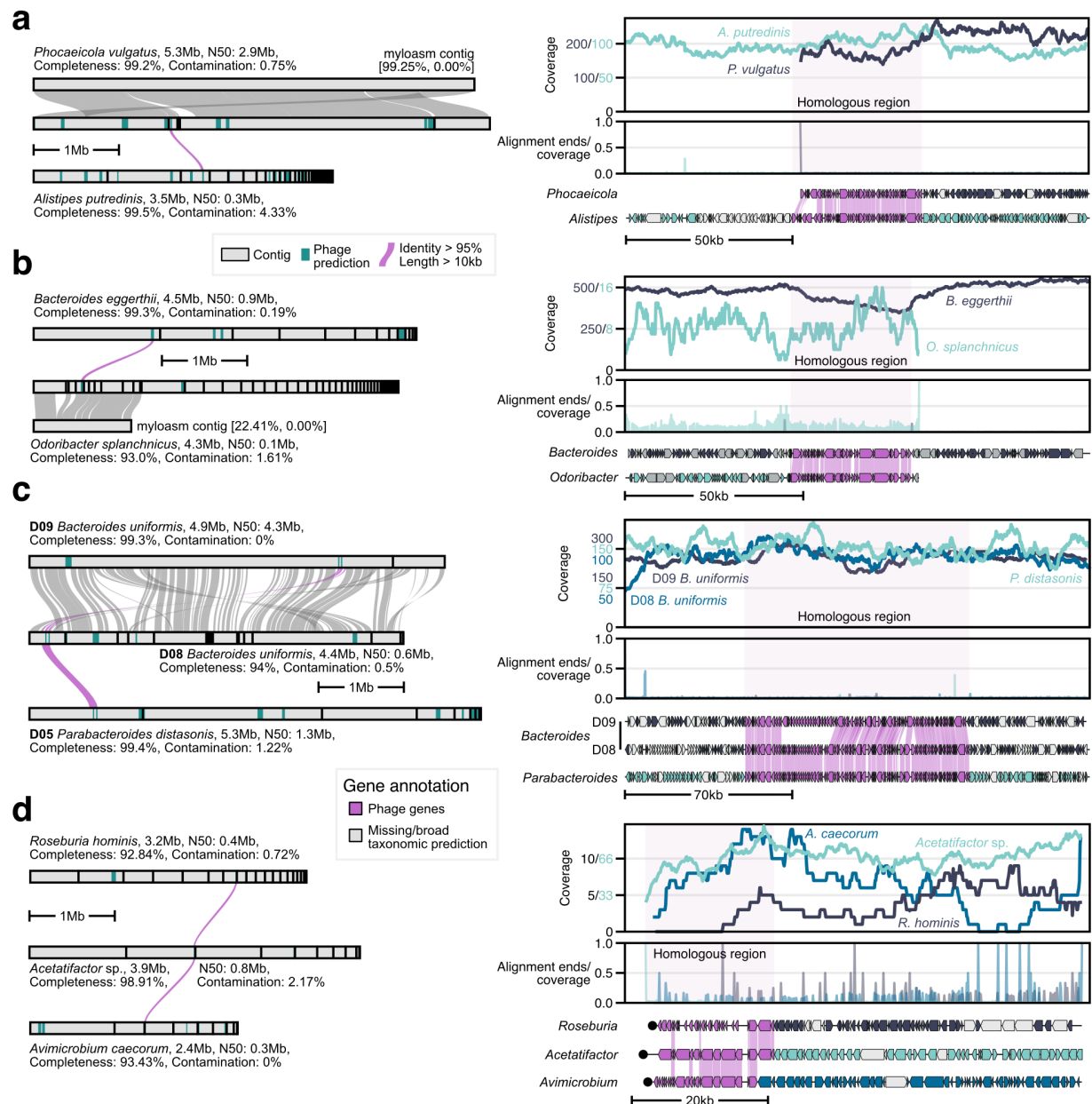


(revised Figure 3)



(revised Supplementary Figure 7)





(revised Supplementary Figure 8)

On the other hand, you state in line 472 "It is important to note, though, that lysogenic host range does not necessarily imply productive host range (the ability to produce infective particles from multiple hosts)". If you perform read analysis for induction (circularity, excision), this would provide compelling evidence for productive infection. I understand that reads implying circularity and excision are not detectable in all prophage regions.

We thank the reviewer for this very insightful suggestion that we had not considered before. We agree that the presence of reads suggesting the presence of circular phage genomes could be additional evidence for productive infection. However, most of the phages integrated into different bacterial families did not overlap a structural variation identified by Sniffles, so we could not determine the exact genomic boundaries of the phage region in question (which is needed for

confident estimation of reads implying the presence of circular genomes). We tried to estimate the exact boundaries from the alignments across contigs and used those boundaries to count the number of reads potentially supporting circular phage genomes. For most of the clusters, we did not find circular reads, which might be an underestimate (since our estimated phage genome borders are potentially incorrect). However, there are two clusters that seem to exhibit evidence of productive infections, as we observe circular reads (see for example the cluster highlighted in the revised Figure 3b). We added the number of circular reads as additional information to the Supplementary Table 2 and extended the discussion section in question.

*Actions taken:*

*We added the number of circular reads to Supplementary Table 2 and modified page 9, lines 311-315 and page 15, lines 512-513.*

Are prophage regions with evidence of excision/circularity more (or less) likely to be dynamically gained/lost?

We would like to thank the reviewer for this question. Indeed, dynamic phages are more likely to overlap SVs detected by Sniffles than stably integrated phages. In the set of dynamic phages, 37% had evidence of excision or circularity compared to 11% of stably integrated phages. Even so, we would have expected most dynamic phages to be represented in the set of SVs detected by Sniffles. Yet, the remaining dynamic phages often overlap SVs that are filtered out by Sniffles for low coverage, highlighting a slight bias towards specificity over sensitivity in our analysis.

*Actions taken:*

*We changed page 16, lines 561-564 to indicate the overlap between dynamic phages and SVs identified by Sniffles as a limitation.*

The prophage boundary analyses, using long read data as ground truth are particularly compelling and important. These and related datasets can help train the next generation of phage/prophage tools to better identify boundaries. Having said this, please add CheckV and Cenote-Taker 3 performance to the analyses related to Fig S6. Relatedly, the Virsorter2 SOP suggests using CheckV for prophage boundary determination: <https://www.protocols.io/view/viral-sequence-identification-sop-with-virsorter2-5qpvoyqebg4o/v3>

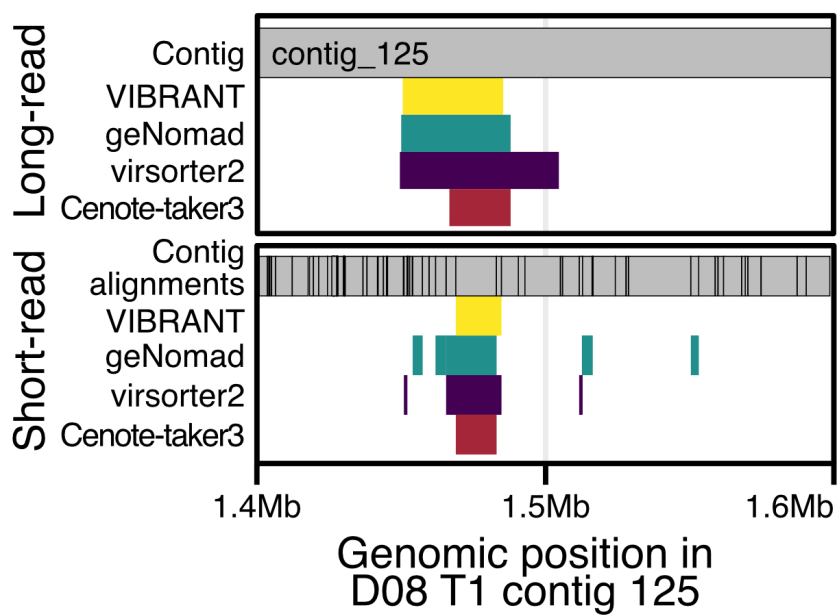
We fully agree with the reviewer in hoping that future efforts to refine prophage prediction tools will benefit from exact boundaries determined by long-read sequencing data. We are particularly grateful for the suggestion to use the CheckV boundary refinement as documented in the virsorter2 SOP to improve the rigor of our analyses. We additionally recognize our oversight of not including Cenote-Taker 3 in our original submission. In the revised manuscript, we have since added Cenote-Taker 3 to all relevant display items (revised Figure 1, Supplementary Figure 2, Supplementary Figure 6) and included the CheckV-adjusted boundaries in our analysis of prophage boundaries.

*Actions taken:*

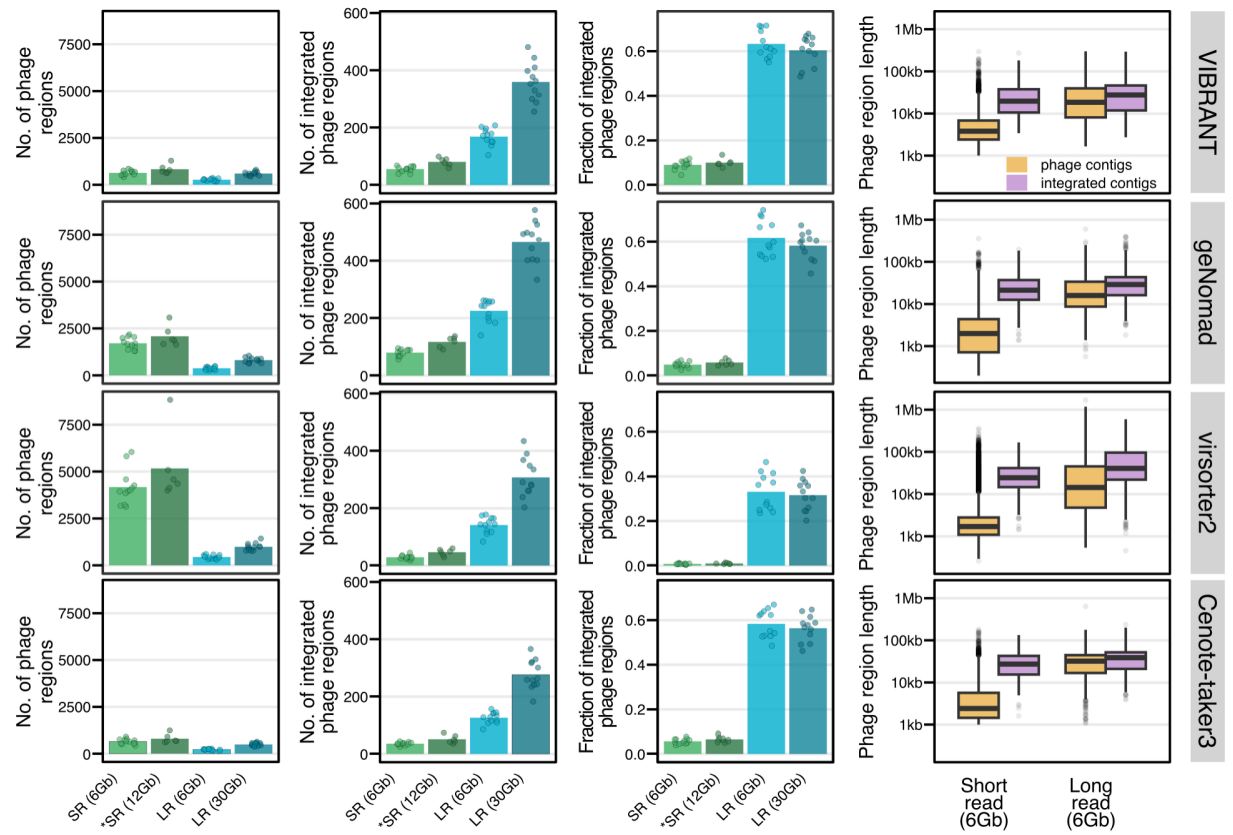
*We ran Cenote-Taker 3 on all assemblies and included the resulting predictions in all relevant figures, tables, and supplementary figures. We additionally used the CheckV boundary*

adjustment for the evaluation of prophage boundaries. The new display items are present in the revised SFig. 6 and the Method section is updated on page 18, lines 648-655.

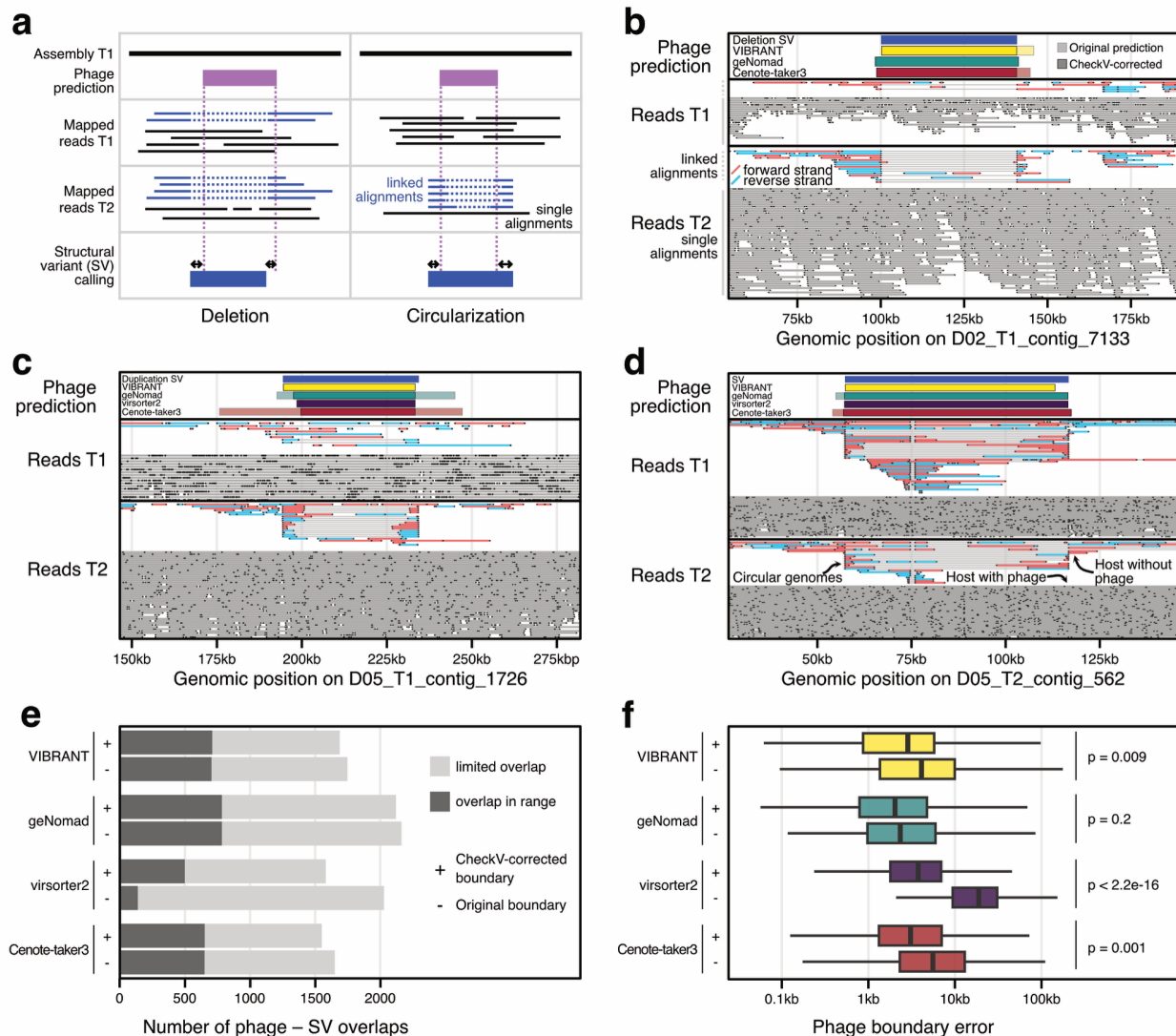
**d**



(revised Figure 1d)



(revised Supplementary Figure 2)



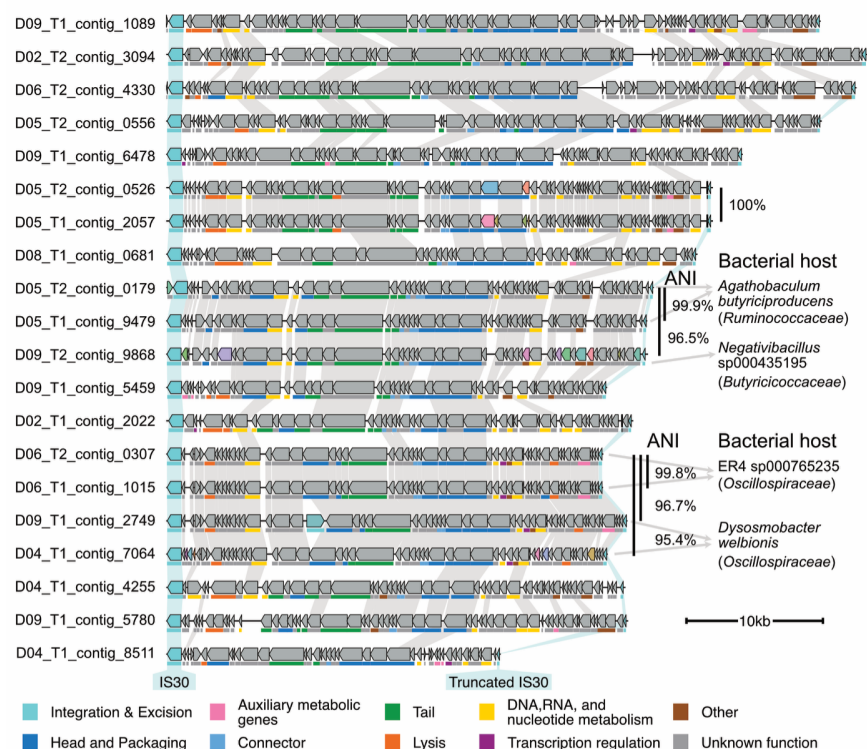
(revised Supplementary Figure 6)

Fig 4B: why is the IS element only annotated on the left end of the phage? Fig 4A and D indicate that there should be a truncated IS element on the right side.

We would like to thank the reviewer for their comment and welcome the opportunity to clarify. The truncated IS element on the right side in the synteny plot was identified by ISEscan. However, the gene synteny plot in Fig. 4b is based on functional annotations derived from the pharokka tool. Pharokka did not annotate the truncated gene at the end of the phage as related to excision/integration, as the main catalytic domain is missing in the truncated genes. However, we manually checked all of the predicted truncated genes with InterProScan and confirmed the presence of an IS30-like helix-turn-helix domain. We appreciate that this distinction between annotation tools was not clear in the original submission and have since revised the figure, the wording of the figure legend, and the accompanying text.

*Actions taken:*

We adjusted panel Fig. 4b to indicate the full length and truncated IS30 in the gene synteny plot (see revised Figure panel below). Additionally, we adjusted the Figure legend to indicate that these annotations were manually confirmed.



(revised Figure 4b)

Analysis repos for the manuscript are not publicly available! Please ameliorate this!

- <https://doi.org/10.5281/zenodo.15192469>
- [https://github.com/bhattlab/long\\_read\\_benchmark](https://github.com/bhattlab/long_read_benchmark)

We apologize for the delayed release of the Zenodo and Github repositories. Both weblinks are accessible without restriction now.