nature portfolio

Peer Review File

Defining the essential genome of diverse phages with phage Tn-seq

Corresponding Author: Professor Peter Fineran

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Loss-of-function screens in bacteriophages have the potential to further our understanding of phage biology and phage-host interactions. Current methods for genetic perturbations of phages, however, are not readily applicable across phages or are not scalable to systematic screens. To address this gap, the authors present Phage Tn-seq, an adaptation of Tn-seq for phages. A key innovation is the use of an anti-CRISPR protein as a selectable marker, which allows for enrichment of transposon mutants on a bacterial host expressing the corresponding CRISPR-Cas system. The authors use Phage Tn-seq to map essential genes in multiple phages, including in jumbo phages that have been difficult to genetically manipulate, and they develop an alternative selection system that further expands the scope of Phage Tn-seq. Finally, the authors demonstrate that their constructs can be repurposed to deliver cargo into phages, opening the door to more efficient phage engineering. The authors include strong validation of the selection strategy and have made all relevant constructs available via Addgene. A particular strength of this work is the application of Phage Tn-seq to poorly characterized phages and to phages with modified DNA, both of which clearly illustrate the potential for the method to catalyze discovery.

Overall, this manuscript is likely to be of high interest to the microbiology research community. Although two other recent pre-prints describe conceptually similar methodologies as Phage Tn-seq, differences in the mutagenesis methodology – including the use of Tn5 in Phage Tn-seq, which has a broader range of insertion sites – make it original and complementary to the other methodologies. In addition, all three groups have applied their methods to distinct phages, revealing novel information about their biology. Nevertheless, there are technical and conceptual issues that need to be addressed prior to publication. These issues primarily relate to a lack of validation of essentiality calls and insufficient consideration of potential confounding effects, as further outlined below.

Major Comments
1. Potential confounding factors for essentiality calls and lack of orthogonal validation
The authors provide validation for their mutagenesis and selection strategy, but the manuscript contains little orthogonal validation of the resulting essentiality calls, which makes it difficult to evaluate their accuracy. Specifically, the authors do not conclusively address three potential factors that may affect the accuracy of the resulting gene essentiality calls, in both JS26 and PCH45. These concerns make it impossible to evaluate the performance of Phage Tn-seq in enabling identification of essential genes with high accuracy. We list these factors below first before making specific suggestions, because the factors are so interconnected that they are impossible to separate.

First, the data included in the supplemental information provide evidence that the reference transposon libraries in JS26 or PCH45 are not sufficiently saturated prior to selection. For JS26, insertions in the late genes are particularly sparse pre-enrichment, and for PCH45, insertions in many of the essential genes are similarly sparse. This lack of saturation limits sensitivity in identification of essential genes and has the potential to cause false positive and false negative results.

Second, the authors use the Bio-Tradis pipeline to classify JS26 and PCH45 genes as essential or non-essential. It is not obvious that Bio-Tradis is the most appropriate pipeline for this purpose. Bio-Tradis has not been validated for phage genomes and appears to explicitly assume a saturated mutant library, which the authors do not have.

Third, the authors discuss polar effects but do not rigorously address if polar effects confound their essentiality calls. Importantly, a recent pre-print documents that polar effects can affect essentiality calls from Tn-seq in phages (https://www.biorxiv.org/content/10.1101/2025.11.23.690004v2). Although the work in the pre-print uses a different transposon (that notably includes a transcriptional terminator, by contrast to the transposon used in this manuscript) and involves different phages, it sets an important precedent for polar effects as potential confounders. In this manuscript, the authors argue against widespread effects of polar effects because they found examples of operons in which upstream non-essential genes contained insertions, whereas downstream essential genes lacked insertions (lines 289-291). This observation suggests an absence of polar effects at those specific loci but does not preclude the existence of polar effects at other loci.

We have the following suggestions to address these issues.
a. We recommend that the authors provide some orthogonal validation of essentiality calls. The manuscript currently contains no such validation, except for one for a JS26 gene from a prior publication (ref. 26). Such validation could consist of targeting a subset of putative non-essential and essential genes by orthogonal methods, such as genetic knockout, Cas13a inhibition, or anti-sense oligos. Validation would be particularly pertinent for the proposed essentiality of all late genes of JS26, which would address both the concern regarding the lack of saturation as well as possible polar effects. It would also be pertinent to confirm the discrepancies between Phage Tn-seq essentiality calls and the data from proteomics of mature virions and conservation across phages. For example, the authors noted that seven structural proteins in JS26 were deemed essential but not detected in mature virions (lines 216-217), and that many proteins in PCH45 were found to be non-essential but were detected in mature virions (lines 379-384) or conserved in Chimalliviridae (lines 422-436).
b. The lack of saturation is a potential concern for researchers seeking to adapt this methodology. Can the authors discuss and/or provide evidence for the source of this lack of saturation? Is it specific to the phages used, an idiosyncrasy of the specific transposon libraries, or a more general downside of the methodology? Can the authors comment if they tried infecting the transposon donor bacterium for a shorter period of time before isolating phages and sequencing insertions? This may be expected to yield a more even starting distribution/more saturated library because it prevents selection against insertions in essential genes from cycles of re-infection. If they did not try this, it would be important to address if they can achieve saturating libraries using such an approach. Notably, the two related studies reported in the pre-prints used the mariner transposon and achieved saturated mutant libraries.
c. The Bio-Tradis script used to assign gene essentiality (tradis_essentiality.R) explicitly states that this analysis requires a saturated mutant library and is not suitable for data sets with low insertion density (line 15). The script produces a number of diagnostic plots which can verify that this condition has been met. Can the authors share these diagnostic plots to establish that their mutant library was sufficiently saturated for analysis by Bio-Tradis?
d. Bio-Tradis was originally developed for Tn-seq in bacteria, whose genomes are larger and more complex. It is unclear whether it is suitable for classifying essential genes in phages. Can the authors comment if essentiality calls are similar between Bio-Tradis and alternative tools, such as TRANSIT or others?
e. It appears that Bio-Tradis quantifies essentiality from the number of unique insertion sites in a gene without considering the sequencing counts at each insertion site. This approach is likely to classify genes as non-essential if sequencing counts of insertion sites decrease but not below a threshold for the classification of presence/absence of an insertion site. Can the authors include supplementary figures showing counts at each insertion site before and after selection to instill further confidence in the classifications? More broadly, a lot of information on essentiality, and fitness effects from insertions, may be encoded from decreased (but non-zero) counts at individual sites. We recommend that the authors consider alternative ways to quantify fitness that are based on these sequencing counts and consider introducing a third category of genes for which insertions impart a partial fitness defect.

2. Discussion of transposon insertion directionality
The authors observe that transposon insertions were enriched in the orientation in which the transcription of the Acr gene and that of the disrupted gene are aligned. They speculate that this enrichment is due to enhanced transcription of the Acr in this orientation as a result of read-through from the native phage promoter (Lines 276-284). But their Acr is driven by a strong promoter already, and the selection appears to work well in general. Have the authors considered the alternative possibility that the insertion bias is the result of selection against insertions in the direction opposite to that of the underlying gene, for example because such insertions may result in head-on collisions of RNA polymerases (owing to the lack of transcriptional terminator on the Acr)?

3. Characterization of AIcrVIA3-LbuCas13a selection system
It would be helpful for readers to have additional information on the novel AIcrVIA3-LbuCas13a selection system that the authors developed. This information is critical to evaluate how robust and generalizable the mutagenesis using this system is.

First, for their initial AcrVIA1-LseCas13a system, the authors quantify selection efficiency by measuring titers of CRISPR-sensitive and -resistant phage in lysates immediately following transposon mutagenesis (Fig. 1E-F). However, with the AIcrVIA3-LbuCas13a system, they take specific plaques picked and propagated following transposon mutagenesis, before measuring the titers of CRISPR-Cas-sensitive and -resistant phage (Fig. 4D-E). This quantifies how resistant each isolated phage mutant is to CRISPR selection, but it does not show the efficiency at which mutants can be selected for from a mixed pool following transposon mutagenesis. Can the authors provide a direct comparison of the selection efficiency between their two systems, for example by applying the protocol used for the AcrVIA1-LseCas13a system to the AIcrVIA3-LbuCas13a system?

Second, the data in Fig. S4D indicate that half of Bas46 plaques (2/4) did not carry a transposon insertion after Tn5-AIcrVIA3 mutagenesis and LbuCas13a selection, which suggests the presence of escape mutants that can survive selection under the AIcrVIA3-LbuCas13a system. Can the authors more rigorously quantify the frequency of escape mutants when wild-type Bas46 is plated on selective hosts expressing LbuCas13a and compare these results to those with the AcrVIA1-LseCas13a system?

Finally, for the PCR screening of LC53 transposon mutants, a band at about the size of the PCR product expected from the transposon is faintly present in the wild-type lysate, which suggests that the primers used may not be entirely specific for the transposon. Can the authors comment on this, repeat the PCR screening with more specific primers, or measure the the frequency of escape mutants for LC53 using the methods described for Bas46?

4. Read mapping to phage genomes
Can the authors explain why in pre-enrichment samples, the percentages of reads that match the transposon are high (~90-96% in Table S1, ~77-85% in UvsX-positive, Table S2), but the percentages of reads that map to the genome are so low (~8-20% in Table S1, ~0.28-0.31% in UvsX-positive, Table S2)? Is it due to carryover and amplification of the transposon mutagenesis plasmid, transposons that have inserted into bacterial DNA, or some technical reason?

Minor Comments

General text

1. The authors include a very brief statement about the two other recent pre-prints that describe methodologies conceptually similar to Phage Tn-seq in their Discussion. It would be beneficial for readers if the authors expanded this section, for example by highlighting implications of the fact that the work described in this manuscript uses a different transposon than the other work.

Introduction

2. In the introduction (Lines 82-83), the authors state: “Here, we developed Tn-seq for phages by leveraging anti-CRISPR (Acr) as a positive selectable marker in the presence of CRISPR-Cas counter-selection.” It would be helpful to cite references here to clarify that this selection strategy has been used before, just not in the context of transposon mutagenesis. These include references 22 and 24 from the bibliography of this manuscript as well as Mayo-Muñoz et al. Viruses 2018 (https://doi.org/10.3390/v10120695).

Figure 1

3. The authors pre-induced the transposase in the bacterial host for 2 h prior to phage infection. This appears to be a critical experimental parameter. Can the authors comment on how they chose this?
4. Figure 1: The caption is missing “Figure 1”. In addition, please consider including the controls for the PCR plaque assay, which are shown in Figure S1C, in Figure 1G.
5. Lines 134-136: it is unclear what the clause “for which it is currently the sole known genus representative” refers to, please adjust.
6. Line 161: duplicated “)”.

Figures 2/3

7. The authors include biological replicates in their mutagenesis experiments, which is excellent, but the authors generally just merge results from the replicates (Fig. 2A, S2A, 3D, S3E). This makes visualization easier but makes it difficult to assess variation across replicates. Can the authors provide a measure of this variation, using correlation metrics or by displaying data of individual replicates side by side in the supplement?
8. For the virion proteomics data, the authors collapse the data into a presence/absence score for each protein (Fig. 2B, 3E). Would it be feasible to show a metric of confidence for detection rather than a binary score? This confidence metric could build on the extensive data shown in Extended Data Table 3.
9. For the legends of Figures 2B and 3E, please include an explanation for the color scale for the “Proteomics” row.
10. Figure 2D has a typo in the spelling of “relative”. The legend also is in a different font than the remainder of the figure. Please also provide a label on the y-axis and consider displaying the 3 replicates side by side, for both pre- and post-selection. Finally, please clarify in the caption which genes were considered to be the “hot-spot region” and were excluded from this analysis.
11. In line 279, the authors conclude that insertion orientation “…bias was not observed for genes prior to counter-selection by Cas13”. It would be more accurate to note that this is true for genes outside the “hot-spot region”.
12. In their figures of the insertion site sequencing counts across the genome (e.g. Figures 2E, S3H), the authors cap the axis at 40 counts. Can the authors explain this choice? Is there any additional information encoded in higher counts?
13. It would be informative to have plots depicting transposon orientation preferences pre-selection, akin to those shown in Figure 2E.

Figure 4

14. Figure 4 only includes panels A and B, but the text makes references to panels C-E. Please adjust.
15. For Figure 4E, please include information on replicates and and standard deviation, as in Figures 1D-F.
16. Line 491: please add the reference from which AIcrIVA3 is derived.
17. For Figure S3D-E, the caption for panel D is missing. The caption labelled D is actually for panel E.
18. In the caption for Figure S4, it appears that there is a typo in the name of the transposon delivery plasmid, which is referred to as pPF4881 for panel C, but as pPF4481 for panel D.

Methods

19. Line 636: possible typo in “pPFpBAD30”?
20. Lines 639-642: possible typo in the name of plasmid “pPF4881”, which is listed as plasmid pPF4481 in Table S5?
21. Line 704: possible typo in “pPF4324”? “pPF4324” is not listed in Table S5.
22. Line 794: possible typo in “…all primers are listed in Supplementary Table S3.”, which appear to be listed in Supplementary Table S6 instead.
23. Some plasmids are mentioned in the methods or main text but are not described in Table S5, including pPF3892 (Line 625) and pPF1951 (Line 669).
24. Some primers are mentioned in the methods or main text but are not described in Table S6, including PF9094, PF9095 (Line 677), PF7658 (Line 767), PF8715 (Line 768), and the primers described in “Library preparation and sequencing” (PF3140, PF8656, PF8657, PF2926).

Data Availability and Code

The authors provide sufficient information in their “Data availability” section such that readers should be able to access all raw sequencing and proteomics data from this study. The NCBI identifiers provided correctly linked to expected datasets. It would be helpful to include somewhere in the manuscript the identifier for their previously published proteomics dataset for JS26 (PXD029878).

In the “Tn-seq data analysis” section, the authors describe all parameters they used for Bio-Tradis pipeline v.1.4.5 (ref. 19). Bio-Tradis is well-maintained and the authors’ description is sufficiently detailed such that it would be possible to replicate their analysis.

It would be helpful if the authors elaborated on their bioinformatic approach in the section “Hypothetical protein functional

assignments":
1. Which databases were searched and what input parameters were used for analyses with NCBI BLAST+ (v.2.17.0) and HH-suite (v.3.3.0)?
2. How were protein annotations prioritized if conflicting results were obtained from searching by sequence similarity, profile HMMs, or structural homology?

Reviewer #2

(Remarks to the Author)
Kyte et al. developed a transposon mutagenesis tool for phages termed Phage Tn-seq that is broadly applicable and versatile. The tool utilises a novel selection approach using anti-CRISPR proteins embedded into a Tn5 transposon for selection. The study mutagenized modified and unmodified phage genomes that infect Prodigiosinella and focused on a nucleus-forming phage. Finally, the authors utilised this technology to deploy genes to phage genomes that represents an important step forward towards generating diverse phage libraries and tailored designer phages.

The study is an important building block for modern phage biology research. The authors considered many factors that are highly relevant to make the system applicable to non-model phage-host pairs, including to put the transposon and transposase on one plasmid, options for conjugation into non-model hosts, and considered double guide RNAs for CRISPR-based selection. In addition, the study focused on details that allowed to derive more conclusions about essentiality, modes of action of expression profiles, and transcriptional directions. This study and the method will for sure accelerate phage research and provide the field with libraries to test defined hypotheses. In addition, the technology will also allow to boost the design of phages through identification of "safe-harbours" in phage genomes for complementation studies. In summary, the study is very carefully considered, well conducted and represents a blueprint for how to establish transposon mutagenesis for other phage-host pairs that will shape phage research in the future. There are still some major points to be addressed to validate the transposon insertion efficiency and essentiality screen.

Major comments
A genome-wide view of essentiality requires a high saturation of the transposon insertion library prior enrichment/selection. After enrichment, the transposon insertions look very well defined and occur impressively for the essential genes only in intergenic regions, whereas for the non-essential genes the saturation is fairly dense. The authors also look in the non-enriched library. To be absolutely clear, this is very much appreciated! Here, surprisingly, there is nearly no saturation for the essential genes, not in JS26 and also not in the nucleus forming phage, that raises many questions. In Suppl. Fig. 2a, there were no insertions in the majority of essential genes (pre-selection), similar for PCH45 (Suppl. Fig. 3D,E). There can be many reasons for this starting from DNA-binding proteins that block transposition, to modifications, or unfavourable folding of the DNA, or as the authors point out later the time window for transposon insertion that should not be applicable to PCH45. This is critical, as the essentiality of a gene can only be validated through ensuring a saturation at the initial time point and demonstrating depletion in later replication rounds.
(1) The cells were infected at MOI 1, how many replication rounds were possible that could have facilitated a natural selection after transposon insertions. The method section mentions that cells were harvested after 18 h post infection, but unlikely the replication cycle is so long, usually 1-2 h, so how many replication rounds were possible? Can it be that many phage replication rounds occurred that resulted in natural selection that depleted insertions in essential genes in the library before enrichment? How do the lysis curves look, is there complete lysis? The authors should repeat the experiment with a high MOI and harvesting shortly after the first round of replication followed by sequencing to avoid any natural selection for JS26 and PCH45. This is critical to validate that transposon insertion was indeed efficient in the proposed essential genes and to claim essentiality.

(2) After the enrichment step (Fig. 1F), the control experiment without selection (WT) shows about 1-2 log10 less phage plaques compare to the pre-enrichment (Fig. 1E). Does the insertion of the transposon add a disadvantage to the phage and what are the consequences for interpretation?

(3) Surprisingly, the number of unique insertions increases by about 5-6-fold after enrichment, where do these additional unique insertions come from through enrichment, should these not be also present in the initial pre-enrichment library? Is this a coverage, sequencing depth effect? Please discuss this in the results section.

(4) The result section (p. 6) summarising the distribution of insertions is misleading, the ~4k insertions if distributed on the whole genome would occur every 17 nt, but here the majority of insertions is only in the first part of the genome, that appears to be injected earlier, as discussed in the next section, please discuss this limitation in a transparent way right away in the results section. In addition, take into account also other explanations from point (1).

The claim that Phage Tn-seq works for phages with (hyper)modified genomes is a strong one and very much required for the broad applicability of this method for general phage biology as many phages share modified genomes, hence important for the method. In this case Tn5 inserts randomly and wouldb e also affected by cytosine modifications, unlike Himar1. Unfortunately, there is not much data provided on the LC53 and Bas46 case in the manuscript that would demonstrate efficient transposon insertion, as claimed in the abstract. In Fig. 4B, the Tn+ plaque showed 4-5 magnitudes less PFUs compared to the wt lysate at (-) condition, similar for LC53, this is a deleterious effect for the library, not comparable to J26 and PCH45, please comment on this. The authors do show that insertions can occur, but the efficiency of transposon mutagenesis should be evaluated for LC53 in a similar depth as for the other Prodigiosinella phages, or it must be discussed that it works but with major limitations related to efficiency.

In the here used transposon, the anti-CRISPR gene is not transcribed from a specific promoter but basically by readthrough, that allows for the analysis of directionality, as done, but at the same time also limits the selection via anti-CRISPR proteins towards highly expressed genes, as discussed. This can also lead to challenges related to the interpretation of essentiality. The data was correlated to proteomics and conservation to validate the outcomes, that matched for the majority of the cases.
(1) But, would it be not more straight forward to correlate this also to the mRNA levels, and include this also as a factor for benchmarking essentiality? Transcriptomics has a much bigger dynamic detection range, and it would allow to understand which expression of the anti-CRISPR gene is required for the negation of the CRSIRP-based selection step. mRNA levels could be also correlated to the insertion index. How do the expression data in Suppl. Fig. 2C correlate with the insertion index pre-enrichment? This should be also included in the interpretation of essentiality, here also the nucleus-forming phage data could be of value as there are many more non-essential genes well distributed in the genome.

(2) In general, it should be also discussed that this bias could be overcome by utilising early phage-derived and strongly expressed promoters for the expression of the anti-CRISPR protein.

LbuCas13 with AIcrVIA3 was here introduced with the idea to use it in combination with the previous LseCas13 AcrVIA1 system. It would be consequently also important to show this, e.g. by delivery of a second reporter in any of the here used phages, e.g. to PCH45::Tn5-mEGFP-AcrVIA1 insert an AIacr via transposons or vice versa, or in another species.

Minor comments

Curiously, in Suppl. Fig. 4B, the GFP is not uniformly distributed as in Fig. 4A and it would be expected for a single protein. Could a read-trough in frame be introduced by transposon insertion that results in a phage-GFP fusion protein? Was there a stop codon inserted in each frame before the GFP gene? This would be required for gene delivery to phage genomes. For the transposon it would not make much of a difference, but it would be still better to have it not fused by coincidence to another protein that could introduce bias. As a remark, GFP can also aggregate at stress. Please clarify on the plasmid/transposon design related to this point, also in the method section.

To show also applicability of Phage Tn-seq for the nucleus-forming phage, the transposase was fused to a phage protein for import into the phage nucleus and the authors claim that this boosted the efficiency. As it is anyway interesting why it worked out at all without fusion to recA, this is an important point to understand nucleus-forming phage biology.
(1) Suppl. Fig. 3G shows that this is only true for 2/3 replicates, and B showed in general less phage titre, please comment on this, and extend the sample size. This will solidify if the requirement for import into the phage nucleus for efficient mutation is true.

(2) In addition, these lysates were already enriched but why is there then still in 2/3 cases ~2 log difference in Suppl. Fig. 3G. Any library after enrichment should reach similar titres as the mutagenized phages will replicate. Was this checked on a lysate prior enrichment?

(3) Suppl. Fig. 3E shows the pre-enrichment transposon insertions for the tnpA experiment (l. 322). But due to low saturation, tnpA was fused to recA (l. 327 ff., Fig. 3D), but the pre-enrichment library is not shown anywhere, please show the pre-enrichment library for Fig. 3D in the Suppl. Fig. 3.

Please discuss if there are any limitations related to phages that degrade the host DNA and henceforth the transposon.

Please discuss if there are any limitation related to phages that encode anti-CRISPR proteins for the selection step.

As a general remark, it would boost the accessibility of this method to include quantitative PCR readouts for the general estimation of insertion saturation and for specific targeted loci to test for their depletion without the requirement to go for sequencing.

l. 18: one CRISPR too much or rephrase it to split the acr and the CRISPR.

l. 120: Reference 22 did not make use of Acrs.

l. 127: the “tightly inducible” araBAD promoter is in mine and other experience not that tight, this is a bit misleading, please rephrase, and make aware about potential issues and ways out in case of toxicity.

l. 131: Plasmids are not available on Addgene for now, please list the identifiers in the manuscript. Very much appreciated to share the plasmids on Addgene!

Suppl. Fig. 3 legend, E is missing.

l. 318: This sequenced library, was it harvested before or after enrichment? This is critical to be comparable to the next section, please clarify in the text.

l. 324: The fact that transposon mutagenesis works without specific localization to the phage nucleus is more than notable. As it is also described that plasmid DNA can recombine with the phage genome, could it be that the transposase/transposon move inside via DNA? This would be worth mentioning in the discussion.

The legend of Fig. 4 contains still elements that are moved to Suppl. Fig. 4, please correct this and update the figure.

Reviewer #3

(Remarks to the Author)
All of my comments have been integrated into the other reviewer's submission.

Decision Letter:

24th February 2026

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Professor Fineran,

Thank you for your patience while your manuscript "High-throughput transposon mutagenesis defines the essential genome of diverse phages" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, the referees brought up several overlapping concerns that will require additional data or analysis. First, there are potential confounding factors for essentiality and additional validation approaches are needed here. Second, both referees agree that you should better characterize the AIcrVIA3-LbuCas13a selection system (and discuss whether/how it could be used in other species). Reviewer #1 also notes a potential issue with disparity in read mapping to phage genomes--more explanation is needed in this regard. Finally, Reviewer #2 noted that the claims about the utility of this approach for phages with hyper-modified genomes needs more data to back this up.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

If revising your manuscript:

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* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months).

In the meantime we hope that you find our referees' comments helpful.

Yours sincerely,

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Reviewer Expertise:

Referee #1: genetics and genomics, CRISPR approaches, phage defense
Referee #2: functional genomics, phage biology
Referee #3: co-reviewer with R1

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

Loss-of-function screens in bacteriophages have the potential to further our understanding of phage biology and phage-host interactions. Current methods for genetic perturbations of phages, however, are not readily applicable across phages or are not scalable to systematic screens. To address this gap, the authors present Phage Tn-seq, an adaptation of Tn-seq for phages. A key innovation is the use of an anti-CRISPR protein as a selectable marker, which allows for enrichment of transposon mutants on a bacterial host expressing the corresponding CRISPR-Cas system. The authors use Phage Tn-seq to map essential genes in multiple phages, including in jumbo phages that have been difficult to genetically manipulate, and they develop an alternative selection system that further expands the scope of Phage Tn-seq. Finally, the authors demonstrate that their constructs can be repurposed to deliver cargo into phages, opening the door to more efficient phage engineering. The authors include strong validation of the selection strategy and have made all relevant constructs available via Addgene. A particular strength of this work is the application of Phage Tn-seq to poorly characterized phages and to phages with modified DNA, both of which clearly illustrate the potential for the method to catalyze discovery.

Overall, this manuscript is likely to be of high interest to the microbiology research community. Although two other recent pre-prints describe conceptually similar methodologies as Phage Tn-seq, differences in the mutagenesis methodology – including the use of Tn5 in Phage Tn-seq, which has a broader range of insertion sites – make it original and complementary to the other methodologies. In addition, all three groups have applied their methods to distinct phages, revealing novel information about their biology. Nevertheless, there are technical and conceptual issues that need to be addressed prior to publication. These issues primarily relate to a lack of validation of essentiality calls and insufficient consideration of potential confounding effects, as further outlined below.

Major Comments
1. Potential confounding factors for essentiality calls and lack of orthogonal validation
The authors provide validation for their mutagenesis and selection strategy, but the manuscript contains little orthogonal validation of the resulting essentiality calls, which makes it difficult to evaluate their accuracy. Specifically, the authors do not

conclusively address three potential factors that may affect the accuracy of the resulting gene essentiality calls, in both JS26 and PCH45. These concerns make it impossible to evaluate the performance of Phage Tn-seq in enabling identification of essential genes with high accuracy. We list these factors below first before making specific suggestions, because the factors are so interconnected that they are impossible to separate.

First, the data included in the supplemental information provide evidence that the reference transposon libraries in JS26 or PCH45 are not sufficiently saturated prior to selection. For JS26, insertions in the late genes are particularly sparse pre-enrichment, and for PCH45, insertions in many of the essential genes are similarly sparse. This lack of saturation limits sensitivity in identification of essential genes and has the potential to cause false positive and false negative results.

Second, the authors use the Bio-Tradis pipeline to classify JS26 and PCH45 genes as essential or non-essential. It is not obvious that Bio-Tradis is the most appropriate pipeline for this purpose. Bio-Tradis has not been validated for phage genomes and appears to explicitly assume a saturated mutant library, which the authors do not have.

Third, the authors discuss polar effects but do not rigorously address if polar effects confound their essentiality calls. Importantly, a recent pre-print documents that polar effects can affect essentiality calls from Tn-seq in phages (https://www.biorxiv.org/content/10.1101/2025.11.23.690004v2). Although the work in the pre-print uses a different transposon (that notably includes a transcriptional terminator, by contrast to the transposon used in this manuscript) and involves different phages, it sets an important precedent for polar effects as potential confounders. In this manuscript, the authors argue against widespread effects of polar effects because they found examples of operons in which upstream non-essential genes contained insertions, whereas downstream essential genes lacked insertions (lines 289-291). This observation suggests an absence of polar effects at those specific loci but does not preclude the existence of polar effects at other loci.

We have the following suggestions to address these issues.
a. We recommend that the authors provide some orthogonal validation of essentiality calls. The manuscript currently contains no such validation, except for one for a JS26 gene from a prior publication (ref. 26). Such validation could consist of targeting a subset of putative non-essential and essential genes by orthogonal methods, such as genetic knockout, Cas13a inhibition, or anti-sense oligos. Validation would be particularly pertinent for the proposed essentiality of all late genes of JS26, which would address both the concern regarding the lack of saturation as well as possible polar effects. It would also be pertinent to confirm the discrepancies between Phage Tn-seq essentiality calls and the data from proteomics of mature virions and conservation across phages. For example, the authors noted that seven structural proteins in JS26 were deemed essential but not detected in mature virions (lines 216-217), and that many proteins in PCH45 were found to be non-essential but were detected in mature virions (lines 379-384) or conserved in Chimalliviridae (lines 422-436).
b. The lack of saturation is a potential concern for researchers seeking to adapt this methodology. Can the authors discuss and/or provide evidence for the source of this lack of saturation? Is it specific to the phages used, an idiosyncrasy of the specific transposon libraries, or a more general downside of the methodology? Can the authors comment if they tried infecting the transposon donor bacterium for a shorter period of time before isolating phages and sequencing insertions? This may be expected to yield a more even starting distribution/more saturated library because it prevents selection against insertions in essential genes from cycles of re-infection. If they did not try this, it would be important to address if they can achieve saturating libraries using such an approach. Notably, the two related studies reported in the pre-prints used the mariner transposon and achieved saturated mutant libraries.
c. The Bio-Tradis script used to assign gene essentiality (tradis_essentiality.R) explicitly states that this analysis requires a saturated mutant library and is not suitable for data sets with low insertion density (line 15). The script produces a number of diagnostic plots which can verify that this condition has been met. Can the authors share these diagnostic plots to establish that their mutant library was sufficiently saturated for analysis by Bio-Tradis?
d. Bio-Tradis was originally developed for Tn-seq in bacteria, whose genomes are larger and more complex. It is unclear whether it is suitable for classifying essential genes in phages. Can the authors comment if essentiality calls are similar between Bio-Tradis and alternative tools, such as TRANSIT or others?
e. It appears that Bio-Tradis quantifies essentiality from the number of unique insertion sites in a gene without considering the sequencing counts at each insertion site. This approach is likely to classify genes as non-essential if sequencing counts of insertion sites decrease but not below a threshold for the classification of presence/absence of an insertion site. Can the authors include supplementary figures showing counts at each insertion site before and after selection to instill further confidence in the classifications? More broadly, a lot of information on essentiality, and fitness effects from insertions, may be encoded from decreased (but non-zero) counts at individual sites. We recommend that the authors consider alternative ways to quantify fitness that are based on these sequencing counts and consider introducing a third category of genes for which insertions impart a partial fitness defect.

2. Discussion of transposon insertion directionality
The authors observe that transposon insertions were enriched in the orientation in which the transcription of the Acr gene and that of the disrupted gene are aligned. They speculate that this enrichment is due to enhanced transcription of the Acr in this orientation as a result of read-through from the native phage promoter (Lines 276-284). But their Acr is driven by a strong promoter already, and the selection appears to work well in general. Have the authors considered the alternative possibility that the insertion bias is the result of selection against insertions in the direction opposite to that of the underlying gene, for example because such insertions may result in head-on collisions of RNA polymerases (owing to the lack of transcriptional terminator on the Acr)?

3. Characterization of AIcrVIA3-LbuCas13a selection system
It would be helpful for readers to have additional information on the novel AIcrVIA3-LbuCas13a selection system that the authors developed. This information is critical to evaluate how robust and generalizable the mutagenesis using this system is.

First, for their initial AcrVIA1-LseCas13a system, the authors quantify selection efficiency by measuring titers of CRISPR-

sensitive and -resistant phage in lysates immediately following transposon mutagenesis (Fig. 1E-F). However, with the AIcrVIA3-LbuCas13a system, they take specific plaques picked and propagated following transposon mutagenesis, before measuring the titers of CRISPR-Cas-sensitive and -resistant phage (Fig. 4D-E). This quantifies how resistant each isolated phage mutant is to CRISPR selection, but it does not show the efficiency at which mutants can be selected for from a mixed pool following transposon mutagenesis. Can the authors provide a direct comparison of the selection efficiency between their two systems, for example by applying the protocol used for the AcrVIA1-LseCas13a system to the AIcrVIA3-LbuCas13a system?

Second, the data in Fig. S4D indicate that half of Bas46 plaques (2/4) did not carry a transposon insertion after Tn5-AIcrVIA3 mutagenesis and LbuCas13a selection, which suggests the presence of escape mutants that can survive selection under the AIcrVIA3-LbuCas13a system. Can the authors more rigorously quantify the frequency of escape mutants when wild-type Bas46 is plated on selective hosts expressing LbuCas13a and compare these results to those with the AcrVIA1-LseCas13a system?

Finally, for the PCR screening of LC53 transposon mutants, a band at about the size of the PCR product expected from the transposon is faintly present in the wild-type lysate, which suggests that the primers used may not be entirely specific for the transposon. Can the authors comment on this, repeat the PCR screening with more specific primers, or measure the the frequency of escape mutants for LC53 using the methods described for Bas46?

4. Read mapping to phage genomes
Can the authors explain why in pre-enrichment samples, the percentages of reads that match the transposon are high (~90-96% in Table S1, ~77-85% in UvsX-positive, Table S2), but the percentages of reads that map to the genome are so low (~8-20% in Table S1, ~0.28-0.31% in UvsX-positive, Table S2)? Is it due to carryover and amplification of the transposon mutagenesis plasmid, transposons that have inserted into bacterial DNA, or some technical reason?

Minor Comments
General text
1. The authors include a very brief statement about the two other recent pre-prints that describe methodologies conceptually similar to Phage Tn-seq in their Discussion. It would be beneficial for readers if the authors expanded this section, for example by highlighting implications of the fact that the work described in this manuscript uses a different transposon than the other work.

Introduction
2. In the introduction (Lines 82-83), the authors state: “Here, we developed Tn-seq for phages by leveraging anti-CRISPR (Acr) as a positive selectable marker in the presence of CRISPR-Cas counter-selection.” It would be helpful to cite references here to clarify that this selection strategy has been used before, just not in the context of transposon mutagenesis. These include references 22 and 24 from the bibliography of this manuscript as well as Mayo-Muñoz et al. Viruses 2018 (https://doi.org/10.3390/v10120695).

Figure 1
3. The authors pre-induced the transposase in the bacterial host for 2 h prior to phage infection. This appears to be a critical experimental parameter. Can the authors comment on how they chose this?
4. Figure 1: The caption is missing “Figure 1”. In addition, please consider including the controls for the PCR plaque assay, which are shown in Figure S1C, in Figure 1G.
5. Lines 134-136: it is unclear what the clause “for which it is currently the sole known genus representative” refers to, please adjust.
6. Line 161: duplicated “)”.

Figures 2/3
7. The authors include biological replicates in their mutagenesis experiments, which is excellent, but the authors generally just merge results from the replicates (Fig. 2A, S2A, 3D, S3E). This makes visualization easier but makes it difficult to assess variation across replicates. Can the authors provide a measure of this variation, using correlation metrics or by displaying data of individual replicates side by side in the supplement?
8. For the virion proteomics data, the authors collapse the data into a presence/absence score for each protein (Fig. 2B, 3E). Would it be feasible to show a metric of confidence for detection rather than a binary score? This confidence metric could build on the extensive data shown in Extended Data Table 3.
9. For the legends of Figures 2B and 3E, please include an explanation for the color scale for the “Proteomics” row.
10. Figure 2D has a typo in the spelling of “relative”. The legend also is in a different font than the remainder of the figure. Please also provide a label on the y-axis and consider displaying the 3 replicates side by side, for both pre- and post-selection. Finally, please clarify in the caption which genes were considered to be the “hot-spot region” and were excluded from this analysis.
11. In line 279, the authors conclude that insertion orientation “…bias was not observed for genes prior to counter-selection by Cas13”. It would be more accurate to note that this is true for genes outside the “hot-spot region”.
12. In their figures of the insertion site sequencing counts across the genome (e.g. Figures 2E, S3H), the authors cap the axis at 40 counts. Can the authors explain this choice? Is there any additional information encoded in higher counts?
13. It would be informative to have plots depicting transposon orientation preferences pre-selection, akin to those shown in Figure 2E.

Figure 4
14. Figure 4 only includes panels A and B, but the text makes references to panels C-E. Please adjust.
15. For Figure 4E, please include information on replicates and and standard deviation, as in Figures 1D-F.
16. Line 491: please add the reference from which AIcrIVA3 is derived.
17. For Figure S3D-E, the caption for panel D is missing. The caption labelled D is actually for panel E.
18. In the caption for Figure S4, it appears that there is a typo in the name of the transposon delivery plasmid, which is referred to as pPF4881 for panel C, but as pPF4481 for panel D.

Methods
19. Line 636: possible typo in “pPFpBAD30”?
20. Lines 639-642: possible typo in the name of plasmid “pPF4881”, which is listed as plasmid pPF4481 in Table S5?
21. Line 704: possible typo in “pPF4324”? “pPF4324” is not listed in Table S5.
22. Line 794: possible typo in “…all primers are listed in Supplementary Table S3.”, which appear to be listed in Supplementary Table S6 instead.
23. Some plasmids are mentioned in the methods or main text but are not described in Table S5, including pPF3892 (Line 625) and pPF1951 (Line 669).
24. Some primers are mentioned in the methods or main text but are not described in Table S6, including PF9094, PF9095 (Line 677), PF7658 (Line 767), PF8715 (Line 768), and the primers described in “Library preparation and sequencing” (PF3140, PF8656, PF8657, PF2926).

Data Availability and Code
The authors provide sufficient information in their “Data availability” section such that readers should be able to access all raw sequencing and proteomics data from this study. The NCBI identifiers provided correctly linked to expected datasets. It would be helpful to include somewhere in the manuscript the identifier for their previously published proteomics dataset for JS26 (PXD029878).
In the “Tn-seq data analysis” section, the authors describe all parameters they used for Bio-Tradis pipeline v.1.4.5 (ref. 19). Bio-Tradis is well-maintained and the authors’ description is sufficiently detailed such that it would be possible to replicate their analysis.
It would be helpful if the authors elaborated on their bioinformatic approach in the section “Hypothetical protein functional assignments”:
1. Which databases were searched and what input parameters were used for analyses with NCBI BLAST+ (v.2.17.0) and HH-suite (v.3.3.0)?
2. How were protein annotations prioritized if conflicting results were obtained from searching by sequence similarity, profile HMMs, or structural homology?

Reviewer #2 (Remarks to the Author):

Kyte et al. developed a transposon mutagenesis tool for phages termed Phage Tn-seq that is broadly applicable and versatile. The tool utilises a novel selection approach using anti-CRISPR proteins embedded into a Tn5 transposon for selection. The study mutagenized modified and unmodified phage genomes that infect Prodigiosinella and focused on a nucleus-forming phage. Finally, the authors utilised this technology to deploy genes to phage genomes that represents an important step forward towards generating diverse phage libraries and tailored designer phages.

The study is an important building block for modern phage biology research. The authors considered many factors that are highly relevant to make the system applicable to non-model phage-host pairs, including to put the transposon and transposase on one plasmid, options for conjugation into non-model hosts, and considered double guide RNAs for CRISPR-based selection. In addition, the study focused on details that allowed to derive more conclusions about essentiality, modes of action of expression profiles, and transcriptional directions. This study and the method will for sure accelerate phage research and provide the field with libraries to test defined hypotheses. In addition, the technology will also allow to boost the design of phages through identification of “safe-harbours” in phage genomes for complementation studies. In summary, the study is very carefully considered, well conducted and represents a blueprint for how to establish transposon mutagenesis for other phage-host pairs that will shape phage research in the future. There are still some major points to be addressed to validate the transposon insertion efficiency and essentiality screen.

Major comments
A genome-wide view of essentiality requires a high saturation of the transposon insertion library prior enrichment/selection. After enrichment, the transposon insertions look very well defined and occur impressively for the essential genes only in intergenic regions, whereas for the non-essential genes the saturation is fairly dense. The authors also look in the non-enriched library. To be absolutely clear, this is very much appreciated! Here, surprisingly, there is nearly no saturation for the essential genes, not in JS26 and also not in the nucleus forming phage, that raises many questions. In Suppl. Fig. 2a, there were no insertions in the majority of essential genes (pre-selection), similar for PCH45 (Suppl. Fig. 3D,E). There can be many reasons for this starting from DNA-binding proteins that block transposition, to modifications, or unfavourable folding of the DNA, or as the authors point out later the time window for transposon insertion that should not be applicable to PCH45. This is critical, as the essentiality of a gene can only be validated through ensuring a saturation at the initial time point and demonstrating depletion in later replication rounds.
(1) The cells were infected at MOI 1, how many replication rounds were possible that could have facilitated a natural selection after transposon insertions. The method section mentions that cells were harvested after 18 h post infection, but unlikely the replication cycle is so long, usually 1-2 h, so how many replication rounds were possible? Can it be that many phage replication rounds occurred that resulted in natural selection that depleted insertions in essential genes in the library before enrichment? How do the lysis curves look, is there complete lysis? The authors should repeat the experiment with a high MOI and harvesting shortly after the first round of replication followed by sequencing to avoid any natural selection for JS26 and PCH45. This is critical to validate that transposon insertion was indeed efficient in the proposed essential genes and to claim essentiality.

(2) After the enrichment step (Fig. 1F), the control experiment without selection (WT) shows about 1-2 log10 less phage plaques compare to the pre-enrichment (Fig. 1E). Does the insertion of the transposon add a disadvantage to the phage and what are the consequences for interpretation?

(3) Surprisingly, the number of unique insertions increases by about 5-6-fold after enrichment, where do these additional unique insertions come from through enrichment, should these not be also present in the initial pre-enrichment library? Is this a coverage, sequencing depth effect? Please discuss this in the results section.

(4) The result section (p. 6) summarising the distribution of insertions is misleading, the ~4k insertions if distributed on the whole genome would occur every 17 nt, but here the majority of insertions is only in the first part of the genome, that appears to be injected earlier, as discussed in the next section, please discuss this limitation in a transparent way right away in the results section. In addition, take into account also other explanations from point (1).

The claim that Phage Tn-seq works for phages with (hyper)modified genomes is a strong one and very much required for the broad applicability of this method for general phage biology as many phages share modified genomes, hence important for the method. In this case Tn5 inserts randomly and wouldb e also affected by cytosine modifications, unlike Himar1. Unfortunately, there is not much data provided on the LC53 and Bas46 case in the manuscript that would demonstrate efficient transposon insertion, as claimed in the abstract. In Fig. 4B, the Tn+ plaque showed 4-5 magnitudes less PFUs compared to the wt lysate at (-) condition, similar for LC53, this is a deleterious effect for the library, not comparable to J26 and PCH45, please comment on this. The authors do show that insertions can occur, but the efficiency of transposon mutagenesis should be evaluated for LC53 in a similar depth as for the other Prodigiosinella phages, or it must be discussed that it works but with major limitations related to efficiency.

In the here used transposon, the anti-CRISPR gene is not transcribed from a specific promoter but basically by readthrough, that allows for the analysis of directionality, as done, but at the same time also limits the selection via anti-CRISPR proteins towards highly expressed genes, as discussed. This can also lead to challenges related to the interpretation of essentiality. The data was correlated to proteomics and conservation to validate the outcomes, that matched for the majority of the cases.
(1) But, would it be not more straight forward to correlate this also to the mRNA levels, and include this also as a factor for benchmarking essentiality? Transcriptomics has a much bigger dynamic detection range, and it would allow to understand which expression of the anti-CRISPR gene is required for the negation of the CRSIRP-based selection step. mRNA levels could be also correlated to the insertion index. How do the expression data in Suppl. Fig. 2C correlate with the insertion index pre-enrichment? This should be also included in the interpretation of essentiality, here also the nucleus-forming phage data could be of value as there are many more non-essential genes well distributed in the genome.

(2) In general, it should be also discussed that this bias could be overcome by utilising early phage-derived and strongly expressed promoters for the expression of the anti-CRISPR protein.

LbuCas13 with AlcrVIA3 was here introduced with the idea to use it in combination with the previous LseCas13 AcrVIA1 system. It would be consequently also important to show this, e.g. by delivery of a second reporter in any of the here used phages, e.g. to PCH45::Tn5-mEGFP-AcrVIA1 insert an Alacr via transposons or vice versa, or in another species.

Minor comments

Curiously, in Suppl. Fig. 4B, the GFP is not uniformly distributed as in Fig. 4A and it would be expected for a single protein. Could a read-trough in frame be introduced by transposon insertion that results in a phage-GFP fusion protein? Was there a stop codon inserted in each frame before the GFP gene? This would be required for gene delivery to phage genomes. For the transposon it would not make much of a difference, but it would be still better to have it not fused by coincidence to another protein that could introduce bias. As a remark, GFP can also aggregate at stress. Please clarify on the plasmid/transposon design related to this point, also in the method section.

To show also applicability of Phage Tn-seq for the nucleus-forming phage, the transposase was fused to a phage protein for import into the phage nucleus and the authors claim that this boosted the efficiency. As it is anyway interesting why it worked out at all without fusion to recA, this is an important point to understand nucleus-forming phage biology.
(1) Suppl. Fig. 3G shows that this is only true for 2/3 replicates, and B showed in general less phage titre, please comment on this, and extend the sample size. This will solidify if the requirement for import into the phage nucleus for efficient mutation is true.

(2) In addition, these lysates were already enriched but why is there then still in 2/3 cases ~2 log difference in Suppl. Fig. 3G. Any library after enrichment should reach similar titres as the mutagenized phages will replicate. Was this checked on a lysate prior enrichment?

(3) Suppl. Fig. 3E shows the pre-enrichment transposon insertions for the tnpA experiment (l. 322). But due to low saturation, tnpA was fused to recA (l. 327 ff., Fig. 3D), but the pre-enrichment library is not shown anywhere, please show the pre-enrichment library for Fig. 3D in the Suppl. Fig. 3.

Please discuss if there are any limitations related to phages that degrade the host DNA and henceforth the transposon.

Please discuss if there are any limitation related to phages that encode anti-CRISPR proteins for the selection step.

As a general remark, it would boost the accessibility of this method to include quantitative PCR readouts for the general estimation of insertion saturation and for specific targeted loci to test for their depletion without the requirement to go for sequencing.

l. 18: one CRISPR too much or rephrase it to split the acr and the CRISPR.

l. 120: Reference 22 did not make use of Acrs.

l. 127: the “tightly inducible” araBAD promoter is in mine and other experience not that tight, this is a bit misleading, please rephrase, and make aware about potential issues and ways out in case of toxicity.

l. 131: Plasmids are not available on Addgene for now, please list the identifiers in the manuscript. Very much appreciated to share the plasmids on Addgene!

Suppl. Fig. 3 legend, E is missing.

l. 318: This sequenced library, was it harvested before or after enrichment? This is critical to be comparable to the next section, please clarify in the text.

l. 324: The fact that transposon mutagenesis works without specific localization to the phage nucleus is more than notable. As it is also described that plasmid DNA can recombine with the phage genome, could it be that the transposase/transposon move inside via DNA? This would be worth mentioning in the discussion.

The legend of Fig. 4 contains still elements that are moved to Suppl. Fig. 4, please correct this and update the figure.

Reviewer #3 (Remarks to the Author):

All of my comments have been integrated into the other reviewer's submission.

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Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Many thanks to the authors for addressing our comments in a comprehensive manner and congratulations on developing this powerful technology. We have no further comments, only a few notes on typos:
1. The caption for Figure 2 does not line up with the panels, it looks like the caption for Figure 2C should refer to panel E, the caption for 2D to panel C, and the caption for 2E to panel D. In addition, there is a reference to Fig. S2E, but it should likely reference Fig. S2F.
2. Line 437: Missing second “)” after “(gp162)”.
3. Line 641: “fundament” should read “fundamental”.
4. Line 976: “.” after “NCBI_Conserved_domains” should be a “,”.
5. References 18 and 31 show up in the list as “(!!! INVALID CITATION !!!)”.
6. We appreciated this sentence in the author reponse: “Although we cannot rule out possible polar effects in some locations, insertions do occur upstream of essential genes in operons.” This is a thoughtful assessment, and we think there would be value to including a sentence that reflects both sides of this assessment in the discussion. That said, the authors should feel free to take or leave this.

Reviewer #2

(Remarks to the Author)
The authors addressed all my points, no further requests.

Minor suggestions:
In addition to Reviewer #1 point 2: A Tn5 transposon-induced anti-sense transcript expression into an essential gene could lead to silencing by RNA-RNA duplex formation and RNase III-induced degradation and apparent essentiality for neighboring genes, optionally to be put up in the discussion, as there was no terminator in the transposon.

Point 3a,b: I do agree that a strong promoter shares benefits, it would be still good to mention that this should match with the RNAP selectivity, e.g. in case of nucleus-forming phages this may cause issues, as phage RNAP is used with specific sigma factors and not the general host RNAPs that are excluded by the nucleus. In essence, I still think, that read-through has a high contribution here that could be mentioned in the discussion.

A statement of caution related to general read coverage and insertion bias would be helpful.

Decision Letter:

16th July 2026

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Professor Fineran, dear Peter,

Thank you for your patience while your manuscript "High-throughput transposon mutagenesis defines the essential genome of diverse phages" was under peer-review at Nature Microbiology. It has now been seen by the 2 referees, whose comments you will find at the end of this email. As you will see, they agree the manuscript has improved, and as such we will be pleased to accept it, in principle. HOWEVER! Before I can do that... I need to send the paper back to you with a quick "revise" decision so that you can re-upload the SI figures as individual ED figures--this is necessary so that our figure screening software can check them, as is our policy for all papers now.

Please don't revise anything else at this stage, and no point by point response is necessary--I'll send along a checklist of final editorial revisions in a week or so, once you get this re-uploaded. Just reupload everything as is, save for adding the SI figs as ED figures of course!! Drop me a line once this is done and I'll get you the official AIP decision letter sent! If you could do this before the end of the week that would be great, as we're now trying to get this caught up to the companion paper! Thanks.

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Please ensure that all correspondence is marked with your Nature Microbiology reference number in the subject line.

Cheers and congrats,

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Reviewers Comments:

Reviewer #1 (Remarks to the Author):

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Reviewer #2 (Remarks to the Author):

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A statement of caution related to general read coverage and insertion bias would be helpful.

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Version 2:

Decision Letter:

Our ref: NMICROBIOL-25124852B

20th July 2026

Dear Dr. Fineran, dear Peter,

Thank you for submitting your revised manuscript "High-throughput transposon mutagenesis defines the essential genome of diverse phages" (NMICROBIOL-25124852B). We'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in a couple of weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Sincerely,

INTERNAL USE ONLY AIP modules required: ["Life Science"]

Version 3:

Decision Letter:

14th August 2026

Dear Professor Fineran,

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Reviewer Comments:

Our responses to reviewer comments are provided in blue text below. Numbering was slightly adjusted for easier readability.

Reviewer #1 (Remarks to the Author):

Loss-of-function screens in bacteriophages have the potential to further our understanding of phage biology and phage-host interactions. Current methods for genetic perturbations of phages, however, are not readily applicable across phages or are not scalable to systematic screens. To address this gap, the authors present Phage Tn-seq, an adaptation of Tn-seq for phages. A key innovation is the use of an anti-CRISPR protein as a selectable marker, which allows for enrichment of transposon mutants on a bacterial host expressing the corresponding CRISPR-Cas system. The authors use Phage Tn-seq to map essential genes in multiple phages, including in jumbo phages that have been difficult to genetically manipulate, and they develop an alternative selection system that further expands the scope of Phage Tn-seq. Finally, the authors demonstrate that their constructs can be repurposed to deliver cargo into phages, opening the door to more efficient phage engineering. The authors include strong validation of the selection strategy and have made all relevant constructs available via Addgene. A particular strength of this work is the application of Phage Tn-seq to poorly characterized phages and to phages with modified DNA, both of which clearly illustrate the potential for the method to catalyze discovery.

Overall, this manuscript is likely to be of high interest to the microbiology research community. Although two other recent pre-prints describe conceptually similar methodologies as Phage Tn-seq, differences in the mutagenesis methodology – including the use of Tn5 in Phage Tn-seq, which has a broader range of insertion sites – make it original and complementary to the other methodologies. In addition, all three groups have applied their methods to distinct phages, revealing novel information about their biology. Nevertheless, there are technical and conceptual issues that need to be addressed prior to publication. These issues primarily relate to a lack of validation of essentiality calls and insufficient consideration of potential confounding effects, as further outlined below.

We thank the reviewer for their enthusiasm for our research and address their raised issues below.

Major Comments

1. Potential confounding factors for essentiality calls and lack of orthogonal validation

   The authors provide validation for their mutagenesis and selection strategy, but the manuscript contains little orthogonal validation of the resulting essentiality calls, which makes it difficult to evaluate their accuracy. Specifically, the authors do not conclusively address three potential factors that may affect the accuracy of the resulting gene essentiality calls, in both JS26 and PCH45. These concerns make it impossible to evaluate the performance of Phage Tn-seq in enabling identification of essential genes with high accuracy. We list these factors below first before making specific suggestions, because the factors are so interconnected that they are impossible to separate.

   First, the data included in the supplemental information provide evidence that the reference transposon libraries in JS26 or PCH45 are not sufficiently saturated prior to selection. For JS26, insertions in the late genes are particularly sparse pre-enrichment, and for PCH45, insertions in many of the essential genes are similarly sparse. This lack of saturation limits sensitivity in identification of essential genes and has the potential to cause false positive and false negative results.

   Second, the authors use the Bio-Tradis pipeline to classify JS26 and PCH45 genes as essential or non-essential. It is not obvious that Bio-Tradis is the most appropriate pipeline for this purpose. Bio-Tradis has not been validated for phage genomes and appears to explicitly assume a saturated mutant library, which the authors do not have.

Third, the authors discuss polar effects but do not rigorously address if polar effects confound their essentiality calls. Importantly, a recent pre-print documents that polar effects can affect essentiality calls from Tn-seq in phages (https://www.biorxiv.org/content/10.1101/2025.11.23.690004v2). Although the work in the pre-print uses a different transposon (that notably includes a transcriptional terminator, by contrast to the transposon used in this manuscript) and involves different phages, it sets an important precedent for polar effects as potential confounders. In this manuscript, the authors argue against widespread effects of polar effects because they found examples of operons in which upstream non-essential genes contained insertions, whereas downstream essential genes lacked insertions (lines 289-291). This observation suggests an absence of polar effects at those specific loci but does not preclude the existence of polar effects at other loci.

We have the following suggestions to address these issues.

a. We recommend that the authors provide some orthogonal validation of essentiality calls. The manuscript currently contains no such validation, except for one for a JS26 gene from a prior publication (ref. 26). Such validation could consist of targeting a subset of putative non-essential and essential genes by orthogonal methods, such as genetic knockout, Cas13a inhibition, or anti-sense oligos. Validation would be particularly pertinent for the proposed essentiality of all late genes of JS26, which would address both the concern regarding the lack of saturation as well as possible polar effects. It would also be pertinent to confirm the discrepancies between Phage Tn-seq essentiality calls and the data from proteomics of mature virions and conservation across phages. For example, the authors noted that seven structural proteins in JS26 were deemed essential but not detected in mature virions (lines 216-217), and that many proteins in PCH45 were found to be non-essential but were detected in mature virions (lines 379-384) or conserved in Chimalliviridae (lines 422-436).

   We agree that orthogonal validation would strengthen the interpretation of the phage Tn-seq method. For JS26 we used dCas9, which has worked effectively in this phage before. We cloned one or two spacers against each of a representative subset of loci (n=12), then performed EOP assays (new **Fig. S2C**). Genes were chosen that were predicted to be non-essential, essential near the cut-off or essential. All essential genes tested were validated as essential using dCas9. Similarly, CRISPRi of all non-essential genes tested demonstrated no phage inhibition – consistent with the Tn-seq predictions. Interestingly, dCas9 targeting of genes deemed essential but near the cut-off by Bio-Tradis did not detectably reduce phage plaquing, suggesting that either these genes are non-essential, the spacers were ineffective or that complete gene disruption (via Tn*5*) is required for the essentiality phenotype.

   For the jumbo phage PCH45 we used dCas13d and cloned two spacers per gene for a subset of loci (n=24), including genes predicted to be essential, non-essential or ambiguous (new **Fig. S3I**). Overall, we observed strong agreement between dCas13d knockdown and Tn-seq predictions. Ten predicted essential genes showed inhibition of phage propagation, except for gp57, which has an insertion index close to “ambiguous” and was predicted as non-essential using Tn5Gaps (see response 1d below). The two dCas13d guides chosen may also have been ineffective. Of 11 predicted non-essential genes tested, dCas13d inhibition aligned with predictions except two (gp174 and gp218) that reduced phage plaquing. As expected, Cas13d knockdown of ambiguous genes revealed both essential and non-essential genes, including gp205 and gp215 – both core chimallin proteins that we also detected in the structural proteomics.

   To address the point regarding library saturation in JS26, we performed deeper sequencing of pre-enrichment samples (new **Fig. S2**). We achieved high genome-wide Tn*5* coverage (~1 insertion per 14 nt on average) indicating that mutations were initially well distributed across the entire genome. Following outgrowth and enrichment, there is specific and strong depletion in loci corresponding to those with predicted essential functions (DNA replication, packaging and virion assembly) – adding further confidence to Bio-Tradis’s assignment of

essentiality and CRISPRi validation. The clustered essentiality organisation seen in phage JS26 is similar to related phages such as lambda (another cos-type phage)[1,2].

We have performed additional work that has increased saturation of PCH45 to one Tn*5* per 22 nt on average (see response 1b below). Evidence that Tn*5* insertions cause minimal polar effects is supported by the PCH45 genome organisation and insertion data, since there are multiple operons containing both essential and non-essential genes. Furthermore, we used dCas13d of three such non-essential genes (gp7, gp50 and gp58) which confirmed non-essential genes upstream of predicted essential genes. We also provide an example (**Fig. S3H**) that shows insertion of transposons in upstream non-essential genes within an operon containing downstream essential genes. JS26 also has non-polar insertions in non-essential genes that are upstream of essential genes in operons (**Fig. 2D**). Although we cannot rule out possible polar effects in some locations, insertions do occur upstream of essential genes in operons.

We thank the reviewer for pointing out that our sentence on the "*seven structural proteins*" was unclear. We have rephrased this to clarify that these are proteins called as essential by phage Tn-seq have predicted roles in phage assembly and release. Therefore, our results were entirely aligned with expectation of not being detected in proteomic analyses of the final particles. We have reworded that section accordingly.

Regarding the differences when comparing Tn-seq essentiality predictions with the structural proteome and genetic conservation: these different datasets are only complementary and supportive (rather than definitive). For example, proteins detected in mature virions are not necessarily essential for infection under the conditions or host strains tested, and conservation does not always equate to essentiality. Interestingly, when we examine the non-essential proteins detected via proteomics in purified PCH45 particles with >50% coverage, most are not conserved genes and therefore may be unique to PCH45 and represent accessory proteins with fitness roles for PCH45 in particular environments or when infecting other bacterial hosts. However, we do detect many non-essential proteins in the proteomic dataset that rank with low % peptide coverage, which might represent co-purification with phage particles rather than being *bona fide* structural components. Specifically, of the 19 proteins with lowest % coverage, 5 are essential and 14 are non-essential. It is difficult to define a precise proteomic cut-off since some of the essential proteins have the lowest coverage and clear structural annotations and conservation.

We have updated the manuscript to address all of the above points and thank the reviewer for the suggestions, which have strengthened the manuscript.

b. The lack of saturation is a potential concern for researchers seeking to adapt this methodology. Can the authors discuss and/or provide evidence for the source of this lack of saturation? Is it specific to the phages used, an idiosyncrasy of the specific transposon libraries, or a more general downside of the methodology? Can the authors comment if they tried infecting the transposon donor bacterium for a shorter period of time before isolating phages and sequencing insertions? This may be expected to yield a more even starting distribution/more saturated library because it prevents selection against insertions in essential genes from cycles of re-infection. If they did not try this, it would be important to address if they can achieve saturating libraries using such an approach. Notably, the two related studies reported in the pre-prints used the mariner transposon and achieved saturated mutant libraries.

The saturation achieved in our enriched libraries is now comparable or better than that reported in the complementary studies and does not suggest a general limitation of the Tn*5*-based phage Tn-seq methodology. It is important to highlight that the pre-enriched libraries are not used for essentiality analyses in our study. The lower sequenced saturation in the original submission relates primarily to the pre-enriched libraries rather than the final post-enriched libraries used for gene essentiality analysis. In the enriched libraries, we obtained

high insertion densities for both phages, with a mean of **one unique insertion per 17 nt for JS26** and one per 58 nt for PCH45. Note that our new increased saturation (see below) has resulted in enriched libraries of **one Tn*5* per 22 nt on average for PCH45**. These values are comparable to those in the phage Mariner Tn-seq studies (e.g. ~ every 42 bp in phiKZ).

Importantly, the saturated Mariner libraries in the related pre-prints were generated from enriched libraries following prolonged mutagenesis, phage replication and Cas13-mediated selection. These datasets are therefore directly comparable to our post-enriched libraries, rather than our pre-enriched samples. Neither study reports sequencing pre-enriched lysates prior to enrichment, making comparison with our pre-enriched libraries unfeasible.

We acknowledge that coverage of the original enriched PCH45 library was lower than JS26. Therefore, we repeated the experiment with several protocol improvements (updated **Methods**), which increased PCH45 titres by at >10-fold in both *tnpA* WT and *uvsX::tnpA* samples and improved unique insertions to ~9,854 (1x Tn5 per 22 nt on average). These new data have been added to the revised manuscript (new **Fig. 3 and Fig. S3**).

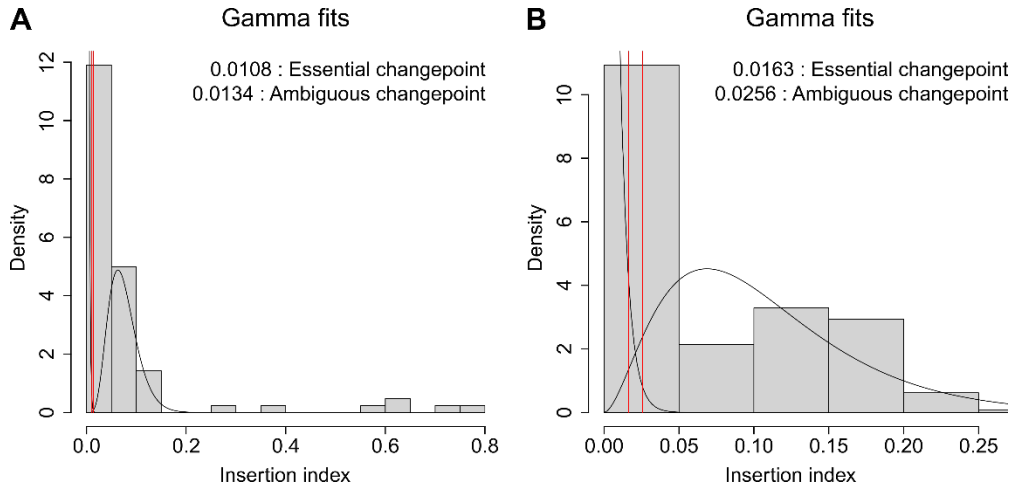
We believe the apparent lack of saturation in the original pre-enriched libraries reflected a technical limitation since the diverse libraries we recovered after Cas13 enrichment must have originated from an initial diverse mutant population. The technical challenge is that mutant phages are initially present at very low abundance (~1 per 10^5 wild-type phages). Consequently, DNA from pre-enriched lysates is dominated by wild-type genomes, making recovery of insertion sites difficult. We have now increased the saturation of the pre-enriched libraries using the improved method (mentioned above). For JS26 this led to an excellent coverage, with 4,465 unique insertion (average density of one Tn5 every 14 nt) (new **Fig. S2A,B**). Importantly, insertions were throughout the genome and within essential genes (as determined following outgrowth with Cas13 selection). PCH45 was technically more challenging to get good pre-enriched libraries, likely due to its smaller burst and longer latent period but we improved these ~10-fold relative to the original manuscript (updated **Table S2**). These pre-enriched PCH45 samples were not needed or used for any analyses.

Since the new Tn*5* insertion data in the pre-enriched JS26 sample demonstrates excellent saturation of essential genes, there is no need to sequencing an earlier timepoint. For PCH45, such samples would suffer from the same fundamental sample limitation we observed for preparation from the 5-hour pre-enriched samples. We have revised the manuscript to include the new data showing higher saturation and clarified that the pre-enriched libraries were included as a proof-of-principle to test whether insertions in essential genes were detected prior to enrichment and were not used for gene essentiality predictions.

c. The Bio-Tradis script used to assign gene essentiality (tradis_essentiality.R) explicitly states that this analysis requires a saturated mutant library and is not suitable for data sets with low insertion density (line 15). The script produces a number of diagnostic plots which can verify that this condition has been met. Can the authors share these diagnostic plots to establish that their mutant library was sufficiently saturated for analysis by Bio-Tradis?

We agree that demonstrating the suitability of Bio-Tradis essentiality analysis is important. As noted above, essentiality was determined using the enriched libraries, which achieved high insertion densities (1 per 17 nt (JS26) or 22 nt (PCH45).

We have included the Bio-Tradis diagnostic plots (**Response Figure 1**), which are designed to assess whether the insertion data are suitable for essentiality calling and set the break-point for essentiality predictions. Our plots differ in distribution relative to bacterial genome datasets (that have a more non-essential than essential genes and more genes overall, providing smoother distributions). As described above (1a and 1b) and in 1d (below), we have multiple lines of evidence that our saturation is sufficient and validated.



Response Figure 1: Bio-Tradis diagnostic plots for JS26 (A) and PCH45 (B) enriched libraries showing a bimodal frequency distribution of insertion indexes (number of inserts per gene divided by gene length). Genes with insertion indexes in the leftmost peak represent those that have none, or very few insertions (essential genes).

d. Bio-Tradis was originally developed for Tn-seq in bacteria, whose genomes are larger and more complex. It is unclear whether it is suitable for classifying essential genes in phages. Can the authors comment if essentiality calls are similar between Bio-Tradis and alternative tools, such as TRANSIT or others?

We agree that since Bio-Tradis was originally developed for bacterial Tn-seq datasets, its suitability for phage genomes was unclear. To address this, we compared the Bio-Tradis essentiality calls with those generated by the Tn5Gaps tool within TRANSIT, a distinct method that identifies essential regions based on statistically significant gaps in insertion coverage. Tn5Gaps evaluates the longest contiguous gap in insertion sites and calculates the probability of observing such a gap by chance using a Gumbel distribution. The Bio-Tradis and Tn5Gaps analyses produced highly concordant essentiality classifications for both phages and we have included in **Extended Data Tables 1 and 2** for phages JS26 and PCH45, respectively. We have added an explanation of the validation to the manuscript and below provide a summary of our findings:

JS26: only two non-essential genes (Gp11 and Gp31) were classed by Tn5gaps as essential in these analyses. Interestingly, these appear to be genes right on the cut-off of essentiality to non-essentiality calls. In agreement, CRISPRi of Gp11 and Gp31 had little or no effect, consistent with the Bio-Tradis non-essential prediction.

PCH45: All genes called as non-essential by Bio-Tradis were confirmed by Tn5Gaps. However, 16 genes were differentially called as non-essential by Tn5Gaps and essential by Bio-Tradis. These genes tended to be closer towards the ambiguous (cut-off) calls made by Bio-Tradis. Additional genes that were predicted as ambiguous by Bio-Tradis were all (except one) called as non-essential by Tn5Gaps. For five of the genes with divergent essentiality predictions between the two tools (gp22, gp44, gp57, gp73 and gp198) we performed dCas13d knockdown which aligned more closely with the Bio-Tradis predictions of essentiality. We also tested four genes called ambiguous by Bio-Tradis using dCas13d knockdown and demonstrated two were essential, whereas two were non-essential. Tn5Gaps predicted these were all non-essential (**Fig. S3I**).

Like any high-throughput screen, there will be some false predictions, particularly near the cut-off/ambiguous class and these may need more care and validation. We have added some text to the manuscript to address this. Overall, the agreement between two independent analytical approaches (and CRISPRi validation) supports the use of Bio-Tradis

for phage Tn-seq. We have now also provided the Tn5Gaps predictions for completeness (updated **Extended Data Tables 1 & 2**).

e. It appears that Bio-Tradis quantifies essentiality from the number of unique insertion sites in a gene without considering the sequencing counts at each insertion site. This approach is likely to classify genes as non-essential if sequencing counts of insertion sites decrease but not below a threshold for the classification of presence/absence of an insertion site. Can the authors include supplementary figures showing counts at each insertion site before and after selection to instill further confidence in the classifications? More broadly, a lot of information on essentiality, and fitness effects from insertions, may be encoded from decreased (but non-zero) counts at individual sites. We recommend that the authors consider alternative ways to quantify fitness that are based on these sequencing counts and consider introducing a third category of genes for which insertions impart a partial fitness defect.

Our primary objective in this study was to define gene essentiality, rather than quantify relative fitness effects of individual insertions. Accordingly, we followed established Tn-seq essentiality approaches, using a saturated mutant library to determine whether genes tolerate transposon insertion. This is distinct from comparative fitness analyses, which are specifically designed to detect changes in insertion abundance across defined conditions. For this reason, we used the standardised Bio-Tradis (& Tn5Gaps) method that relies on unique insertion sites as the metric for essentiality calling from a single sample (not comparing two conditions as is required for fitness analyses). As outlined in responses to comments 1b–d, the density of our libraries and the concordance between independent methods support our approach.

We agree that genes with partial fitness defects represent an interesting biological category for future analyses. However, interpretation of insertion counts in JS26 (and potentially other phages) is complicated by the elevated insertion density observed near the highly expressed early injected portion of the genome. Furthermore, read depth can be influenced by PCR bias or library clustering efficiency. We therefore consider binary essential/non-essential classification by Bio-Tradis to be the most appropriate analysis for the current datasets.

2. Discussion of transposon insertion directionality

a. The authors observe that transposon insertions were enriched in the orientation in which the transcription of the Acr gene and that of the disrupted gene are aligned. They speculate that this enrichment is due to enhanced transcription of the Acr in this orientation as a result of read-through from the native phage promoter (Lines 276-284). But their Acr is driven by a strong promoter already, and the selection appears to work well in general. Have the authors considered the alternative possibility that the insertion bias is the result of selection against insertions in the direction opposite to that of the underlying gene, for example because such insertions may result in head-on collisions of RNA polymerases (owing to the lack of transcriptional terminator on the Acr)?

We agree that the insertion orientation after Cas13 selection could be explained by increased Acr production and / or by collisions in the reverse orientation impairing Acr expression. To recognise this, we now added the following text to the manuscript:

"... following Cas13 counter-selection, insertions were strongly biased in the same orientation as gene transcription (Fig. 2C and Fig. S2E). Because this bias emerged only after Cas13 enrichment, it reflects selection for phages expressing the Acr rather than intrinsic Tn5 insertion preferences. Several non-mutually exclusive mechanisms could explain this orientation bias. Insertions in the same orientation as phage transcription may permit transcriptional read-through from phage promoters, increasing Acr expression, whereas insertions in the opposite orientation could result in RNA polymerase collisions that impede Acr transcription. Both mechanisms would favour phages carrying insertions aligned with gene transcription during Cas13 selection."

3. Characterization of AIcrVIA3-LbuCas13a selection system
It would be helpful for readers to have additional information on the novel AIcrVIA3-LbuCas13a selection system that the authors developed. This information is critical to evaluate how robust and generalizable the mutagenesis using this system is.

   a. First, for their initial AcrVIA1-LseCas13a system, the authors quantify selection efficiency by measuring titers of CRISPR-sensitive and -resistant phage in lysates immediately following transposon mutagenesis (Fig. 1E-F). However, with the AIcrVIA3-LbuCas13a system, they take specific plaques picked and propagated following transposon mutagenesis, before measuring the titers of CRISPR-Cas-sensitive and -resistant phage (Fig. 4D-E). This quantifies how resistant each isolated phage mutant is to CRISPR selection, but it does not show the efficiency at which mutants can be selected for from a mixed pool following transposon mutagenesis. Can the authors provide a direct comparison of the selection efficiency between their two systems, for example by applying the protocol used for the AcrVIA1-LseCas13a system to the AIcrVIA3-LbuCas13a system?

      Our goal in including the AIcrVIA3-LbuCas13a data was not to identify a superior selection system, but rather to demonstrate that we have multiple orthogonal Cas13–anti-CRISPR combinations to enable the generation of double phage mutants (new **Fig. 4C-E**) and to generate and enrich transposon mutant phage populations. We have revised the manuscript to better clarify our aims with the orthogonal AI-Acr system.

      However, as requested, we have now performed experiments identical to those shown in Fig. 1E–F using the AIcrVIA3-LbuCas13a system and have added the data to the manuscript (new **Fig. S4G,H**). These experiments directly demonstrate enrichment of mutant phages from a mixed population following transposon mutagenesis, confirming the AIcrVIA3-LbuCas13a system can be used for mutant selection in the same manner as the AcrVIA1-LseCas13a system.

      We note, however, that care should be taken before drawing a direct quantitative comparison of selection efficiencies between the two systems. This is because selection efficiency is influenced by multiple factors, including the expression/strength of Cas13 orthologue in the bacterial strain tested, spacer sequence, escape frequency and the particular phage or gene targeted. In the current study, the two systems were tested using different spacers and different phages, and we did not perform the same extensive spacer optimisation for the LbuCas13a system as we did for LseCas13a (i.e., involving double spacers to strengthen selection). Instead, we used previously validated single spacer constructs[3], which provides proof-of-principle functionality for LbuCas13a and shows a new orthogonal system can be easily established with little optimisation.

   b. Second, the data in Fig. S4D indicate that half of Bas46 plaques (2/4) did not carry a transposon insertion after Tn5-AIcrVIA3 mutagenesis and LbuCas13a selection, which suggests the presence of escape mutants that can survive selection under the AIcrVIA3-LbuCas13a system. Can the authors more rigorously quantify the frequency of escape mutants when wild-type Bas46 is plated on selective hosts expressing LbuCas13a and compare these results to those with the AcrVIA1-LseCas13a system?

      We have performed further work and added more data assessing the AIcrVIA3-LbuCas13a system, including LC53 and Bas46 phage titres before and after enrichment (new **Fig. S4G, H**) and additional plaque PCRs (new **Fig. S4F**).

      The original negative plaque PCR results for Bas46 were partly due to technical issues during plaque isolation, which were improved by increasing agar overlay concentration (updated in **Methods**). Following optimisation, the proportion of plaques with transposons increased (12/16 for Bas46 and 16/16 for LC53). Therefore, we still observed a few Bas46 escape plaques but this is not unexpected, as the AIcrVIA3-LbuCas13a experiments were intended as a proof-of-principle and used a single previously validated spacer without further optimisation. In contrast, the AcrVIA1-LseCas13a system was extensively optimised (see 3a

above). Given that escape frequency is highly spacer- and phage-dependent, quantification of the escape frequencies using the current datasets would not enable a meaningful comparison between the Lbu and Lse systems. As noted above (point 3a), the AIcrVIA3-LbuCas13a experiments were designed to demonstrate that orthogonal Cas13-Acr pairs can select already mutagenised phages, enabling double mutant generation and expanding the applicability of phage Tn-seq. We have revised the manuscript to clarify this.

c. Finally, for the PCR screening of LC53 transposon mutants, a band at about the size of the PCR product expected from the transposon is faintly present in the wild-type lysate, which suggests that the primers used may not be entirely specific for the transposon. Can the authors comment on this, repeat the PCR screening with more specific primers, or measure the frequency of escape mutants for LC53 using the methods described for Bas46?

   The same methods and primers had been used on LC53 and Bas46. However, there was some non-specific products from the LC53 genome. Therefore, we have repeated the plaque PCR with newly designed primers (PF9691&PF9692 and **Table S6**). The results have been added to a new **Fig. S4F**. As described in our response to 2b we observed 16/16 LC53 plaques contained the transposon.

4. Read mapping to phage genomes

   a. Can the authors explain why in pre-enrichment samples, the percentages of reads that match the transposon are high (~90-96% in Table S1, ~77-85% in UvsX-positive, Table S2), but the percentages of reads that map to the genome are so low (~8-20% in Table S1, ~0.28-0.31% in UvsX-positive, Table S2)? Is it due to carryover and amplification of the transposon mutagenesis plasmid, transposons that have inserted into bacterial DNA, or some technical reason?

   We have now prepared new libraries and re-sequenced our samples using our improved method, which has improved this issue. This enabled increased saturation and reduced the earlier background sequencing of insertions in the transposon plasmid. All new data is provided in updated **Tables S1 and S2** and show that on average we have 80-90% of reads mapping to the JS26 and PCH45 phage genomes. One exception is the pre-enriched PCH45 sequencing, which we have improved to around 2-10% between replicates for the UvsX-TnpA samples. The higher background in the pre-enriched PCH45 samples reflects the challenges with the lower phage yield for this jumbo phage. We have opted to report the pre-enriched PCH45 sequencing for transparency (**Table S2**) but as mentioned above, the this data is not required for any analyses of gene essentiality calling.

Minor Comments

General text

1. The authors include a very brief statement about the two other recent pre-prints that describe methodologies conceptually similar to Phage Tn-seq in their Discussion. It would be beneficial for readers if the authors expanded this section, for example by highlighting implications of the fact that the work described in this manuscript uses a different transposon than the other work.

   We have expanded the discussion accordingly.

Introduction

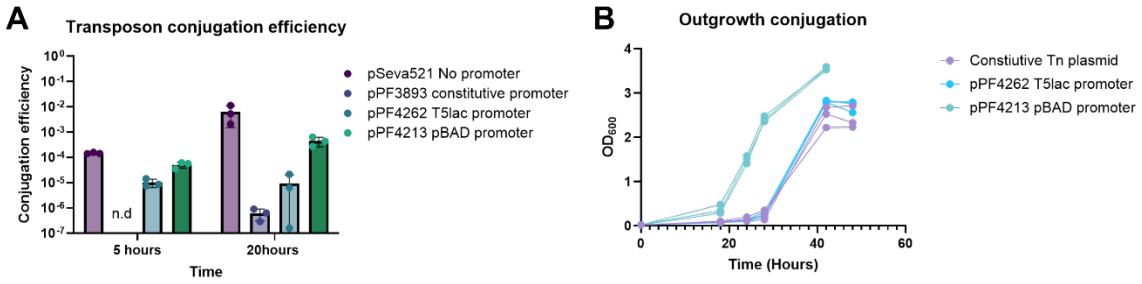
2. In the introduction (Lines 82-83), the authors state: “Here, we developed Tn-seq for phages by leveraging anti-CRISPR (Acr) as a positive selectable marker in the presence of CRISPR-Cas counter-selection.” It would be helpful to cite references here to clarify that this selection strategy has been used before, just not in the context of transposon mutagenesis. These include references 22 and 24 from the bibliography of this manuscript as well as Mayo-Muñoz et al. Viruses 2018 (https://doi.org/10.3390/v10120695).

We have added the references accordingly.

Figure 1

3. The authors pre-induced the transposase in the bacterial host for 2 h prior to phage infection. This appears to be a critical experimental parameter. Can the authors comment on how they chose this?

   The 2 h induction was selected after optimising transposase expression in the host. Initially, the transposase was expressed from a constitutive promoter but resulted in host toxicity, likely due to mutagenesis of the bacterial genome prior to phage infection. We therefore explored inducible systems to minimise toxicity while maintaining efficient phage mutagenesis. Specifically, we tested the T5-lac IPTG-inducible and pBAD arabinose inducible promoters. **Response Figure 2** shows constitutive transposase expression was toxic to the host as measured by conjugation efficiency and bacterial outgrowth, while the T5-lac promoter still resulted in considerable growth inhibition. In contrast, the pBAD system provided sufficient control of transposase expression and largely eliminated toxicity while maintaining efficient phage mutagenesis. Using this system, a 2 h induction period was sufficient to accumulate sufficient transposase prior to phage infection while avoiding prolonged exposure of the bacterial host to transposition activity. This induction time was consistent with published expression kinetics for the pBAD promoter[4]. We have added this rationale to the Methods section.



Response Figure 2: (A) Conjugation efficiencies (for 5 or 20 h) of plasmids encoding the *tnpA* transposase and Tn5 transposon with a constitutive promoter (pPF3893), a T5lac promoter (pPF4262) or a pBAD promoter (pPF4213) regulating *tnpA* expression, compared with an empty vector control with no promoter present (pSEVA531). (B) Outgrowth following conjugation of bacterial strains from (A) containing the transposon plasmids with different promoters driving *tnpA* expression.

4. Figure 1: The caption is missing “Figure 1”. In addition, please consider including the controls for the PCR plaque assay, which are shown in Figure S1C, in Figure 1G.

   The missing “Figure 1” caption as well as the PCR controls shown in **Fig. S1C** have been added to **Fig. 1.**

5. Lines 134-136: it is unclear what the clause “for which it is currently the sole known genus representative” refers to, please adjust.

   There is now evidence that there are other *Prodigiosinella* strains, so the text has been adjusted accordingly.

6. Line 161: duplicated “)”.

   The duplicate “)” has been removed.

Figures 2/3

7. The authors include biological replicates in their mutagenesis experiments, which is excellent, but the authors generally just merge results from the replicates (Fig. 2A, S2A, 3D, S3E). This makes visualization easier but makes it difficult to assess variation across replicates. Can the authors provide a measure of this variation, using correlation metrics or by displaying data of individual replicates side by side in the supplement?

We have updated **Fig. 2A, S2A, 3D and S3E** to now show all biological replicates. We thank the reviewer for this suggestion as it now clearly shows the variation and reproducibility of our replicate samples. It is also worth noting that from an analysis perspective, Bio-Tradis does not use replicate data but the overall unique insertion information.

8. For the virion proteomics data, the authors collapse the data into a presence/absence score for each protein (Fig. 2B, 3E). Would it be feasible to show a metric of confidence for detection rather than a binary score? This confidence metric could build on the extensive data shown in Extended Data Table 3.

   To provide a more quantitative visual summary in the main figures, we have updated **Fig. 2B and 3E** to display % peptide sequence coverage rather than simply presence/absence. We selected coverage because it provides an intuitive measure of detection and is easily visualised on a heatmap alongside our other data types, while being less influenced by protein length than metrics such as number of identified peptides or PSMs. All proteomic metrics remain available in **Extended Data Table 3** and in the JS26 publication[5].

9. For the legends of Figures 2B and 3E, please include an explanation for the color scale for the "Proteomics" row.

   This has now been updated in response to the changes to point 8.

10. Figure 2D has a typo in the spelling of "relative". The legend also is in a different font than the remainder of the figure. Please also provide a label on the y-axis and consider displaying the 3 replicates side by side, for both pre- and post-selection. Finally, please clarify in the caption which genes were considered to be the "hot-spot region" and were excluded from this analysis.

    This had been updated in the manuscript and supplement.

11. In line 279, the authors conclude that insertion orientation "…bias was not observed for genes prior to counter-selection by Cas13". It would be more accurate to note that this is true for genes outside the "hot-spot region".

    The wording has been adjusted accordingly and has benefitted from our higher saturation pre-enrichment libraries obtained for phage JS26.

12. In their figures of the insertion site sequencing counts across the genome (e.g. Figures 2E, S3H), the authors cap the axis at 40 counts. Can the authors explain this choice? Is there any additional information encoded in higher counts?

    This was done for improved readability as lower transposon insertion numbers are hard to visualise otherwise. We have added the explanation to the figure legends.

13. It would be informative to have plots depicting transposon orientation preferences pre-selection, akin to those shown in Figure 2E.

    We have now added a figure the same as **Fig. 2E** as a new **Fig. S2F** that shows the insertion orientation plots pre-enrichment. We have also updated **Fig. S2E** to show this data quantified for each separate biological replicate.

14. Figure 4 only includes panels A and B, but the text makes references to panels C-E. Please adjust.

    Panels C-E are now added to Figure 4.

15. For Figure 4E, please include information on replicates and standard deviation, as in Figures 1D-F.

    We have repeated the experiment and updated the figure including standard deviations. Information regarding the replicates has been added to the figure legend.

16. Line 491: please add the reference from which AIcrIVA3 is derived.

    We have added the reference.

17. For Figure S3D-E, the caption for panel D is missing. The caption labelled D is actually for panel E.

    We have added the figure description for panel D.

18. In the caption for Figure S4, it appears that there is a typo in the name of the transposon delivery plasmid, which is referred to as pPF4881 for panel C, but as pPF4481 for panel D.

    We have corrected the plasmid number in the figure description for panel C.

Methods

19. Line 636: possible typo in “pPFpBAD30”?

    “pPF” has been removed.

20. Lines 639-642: possible typo in the name of plasmid “pPF4881”, which is listed as plasmid pPF4481 in Table S5?

    Plasmid number “pPF4881” was corrected to “pPF4481”.

21. Line 704: possible typo in “pPF4324”? “pPF4324” is not listed in Table S5.

    Plasmid number “pPF4324” was corrected to “pPF4323”.

22. Line 794: possible typo in “…all primers are listed in Supplementary Table S3.”, which appear to be listed in Supplementary Table S6 instead.

    “Supplementary Table S3” was corrected to “Supplementary Table S6”.

23. Some plasmids are mentioned in the methods or main text but are not described in Table S5, including pPF3892 (Line 625) and pPF1951 (Line 669).

    Missing plasmids were added to plasmid list

24. Some primers are mentioned in the methods or main text but are not described in Table S6, including PF9094, PF9095 (Line 677), PF7658 (Line 767), PF8715 (Line 768), and the primers described in “Library preparation and sequencing” (PF3140, PF8656, PF8657, PF2926).

    All missing primers were added to the primer list.

Data Availability and Code

25. The authors provide sufficient information in their “Data availability” section such that readers should be able to access all raw sequencing and proteomics data from this study. The NCBI identifiers provided correctly linked to expected datasets. It would be helpful to include somewhere in the manuscript the identifier for their previously published proteomics dataset for JS26 (PXD029878).

    A link to the previously published JS26 proteomics dataset was added to the “Data availability” section.

In the “Tn-seq data analysis” section, the authors describe all parameters they used for Bio-Tradis pipeline v.1.4.5 (ref. 19). Bio-Tradis is well-maintained and the authors’ description is sufficiently detailed such that it would be possible to replicate their analysis.

It would be helpful if the authors elaborated on their bioinformatic approach in the section “Hypothetical protein functional assignments”:

1. Which databases were searched and what input parameters were used for analyses with NCBI BLAST+ (v.2.17.0) and HH-suite (v.3.3.0)?

   NCBI BLAST+ (v.2.17.0) was searched using protein sequencings against the ClusteredNR data base and HH-suite (v.3.3.0) used hhpred to search protein databases PHROGs, NCBI_Conserved_domains. Pfam-A and PDB_mmCIF70

2. How were protein annotations prioritized if conflicting results were obtained from searching by sequence similarity, profile HMMs, or structural homology?

   Overall, very few conflicting annotation results were observed. Most discrepancies occurred for proteins where none of the identified matches showed particularly strong support. Annotations were therefore assigned based on the overall strength of evidence, with priority given to hits showing the highest confidence, as determined by probability scores, E-values, alignment coverage, and consistency across multiple databases. Where evidence remained weak or inconclusive, proteins were annotated as hypothetical proteins.

Reviewer #2 (Remarks to the Author):

Kyte et al. developed a transposon mutagenesis tool for phages termed Phage Tn-seq that is broadly applicable and versatile. The tool utilises a novel selection approach using anti-CRISPR proteins embedded into a Tn5 transposon for selection. The study mutagenized modified and unmodified phage genomes that infect Prodigiosinella and focused on a nucleus-forming phage. Finally, the authors utilised this technology to deploy genes to phage genomes that represents an important step forward towards generating diverse phage libraries and tailored designer phages.

The study is an important building block for modern phage biology research. The authors considered many factors that are highly relevant to make the system applicable to non-model phage-host pairs, including to put the transposon and transposase on one plasmid, options for conjugation into non-model hosts, and considered double guide RNAs for CRISPR-based selection. In addition, the study focused on details that allowed to derive more conclusions about essentiality, modes of action of expression profiles, and transcriptional directions. This study and the method will for sure accelerate phage research and provide the field with libraries to test defined hypotheses. In addition, the technology will also allow to boost the design of phages through identification of “safe-harbours” in phage genomes for complementation studies. In summary, the study is very carefully considered, well conducted and represents a blueprint for how to establish transposon mutagenesis for other phage-host pairs that will shape phage research in the future. There are still some major points to be addressed to validate the transposon insertion efficiency and essentiality screen.

We thank the reviewer for their enthusiasm for our research and address their raised issues below.

Major comments

1. A genome-wide view of essentiality requires a high saturation of the transposon insertion library prior enrichment/selection. After enrichment, the transposon insertions look very well defined and occur impressively for the essential genes only in intergenic regions, whereas for the non-essential genes the saturation is fairly dense. The authors also look in the non-enriched library. To be absolutely clear, this is very much appreciated! Here, surprisingly, there is nearly no saturation for the essential genes, not in JS26 and also not in the nucleus forming phage, that raises many questions. In Suppl. Fig. 2a, there were no insertions in the majority of essential genes (pre-selection), similar for PCH45 (Suppl. Fig. 3D,E). There can be many reasons for this starting from DNA-binding proteins that block transposition, to modifications, or unfavourable folding of the DNA, or as the authors point out later the time window for transposon insertion that should not be applicable to PCH45. This is critical, as the essentiality of a gene can only be validated through ensuring a saturation at the initial time point and demonstrating depletion in later replication rounds.

Please see responses to reviewer #1 point 1a and 1b that addresses the point that we have improved saturation and that the pre-enrichment libraries are not used for essentiality gene calling in the Bio-Tradis pipeline.

a. The cells were infected at MOI 1, how many replication rounds were possible that could have facilitated a natural selection after transposon insertions. The method section mentions that cells were harvested after 18 h post infection, but unlikely the replication cycle is so long, usually 1-2 h, so how many replication rounds were possible? Can it be that many phage replication rounds occurred that resulted in natural selection that depleted insertions in essential genes in the library before enrichment? How do the lysis curves look, is there complete lysis? The authors should repeat the experiment with a high MOI and harvesting shortly after the first round of replication followed by sequencing to avoid any natural selection for JS26 and PCH45. This is critical to validate that transposon insertion was indeed efficient in the proposed essential genes and to claim essentiality.

   Thanks for highlighting an error in our methods section. The 18 h incubation refers only to the enrichment step. The phage infection during the transposon mutagenesis step was performed for 5–6 h, as correctly depicted in **Fig. S1D**. The 18 h duration was mistakenly stated in the Methods section and has now been corrected.

   As described above (Reviewer #1 points 1a and 1b), we now have more highly saturated pre-enrichment libraries that demonstrate the existence of genome-wide mutations prior to the outgrowth/enrichment step, including in essential genes for JS26. Therefore, the need to sample earlier is not required. We agree that multiple rounds of phage replication will occur and that these will select against insertions in essential genes. Indeed, this is the basis of the standard Bio-Tradis/Tn5Gaps essentiality analysis, as mutants carrying insertions in essential genes are unable to complete productive replication and are consequently depleted from the population. As noted earlier the pre-enrichment library is not required or used by these tools for essentiality calls, but it does provide confirmation the transposon insertions were well-distributed prior to phage replication.

b. After the enrichment step (Fig. 1F), the control experiment without selection (WT) shows about 1-2 log10 less phage plaques compare to the pre-enrichment (Fig. 1E). Does the insertion of the transposon add a disadvantage to the phage and what are the consequences for interpretation?

   Although it is possible that some transposon insertions reduce phage fitness, there is a more likely explanation for the reduction in total PFU/ml after Cas13 selection/enrichment. Pre-enrichment and post-enrichment lysates are not directly comparable in terms of total phage titre. Following mutagenesis, the lysate contains unmutagenised wild-type phages and transposon-containing mutants. During enrichment, Cas13 depletes the unmutagenised wild-type phages, while phages carrying the Acr transposon are selectively enriched. Therefore, the reduced titre after enrichment primarily reflects removal of abundant wild-type phages, rather than a general disadvantage caused by transposon insertion.

   Therefore, the most informative comparison is the relative titre of each lysate on non-selective versus selective hosts rather than the absolute titre before vs. after enrichment. Before enrichment, titres differ markedly between these +/-Cas13, consistent with most phages lacking the transposon. After enrichment, titres on +/-Cas13 are more similar, indicating that the lysate is largely composed of transposon-containing phages. We have clarified this in the manuscript.

c. Surprisingly, the number of unique insertions increases by about 5-6-fold after enrichment, where do these additional unique insertions come from through enrichment, should these not be also present in the initial pre-enrichment library? Is this a coverage, sequencing depth effect? Please discuss this in the results section.

See our responses (Reviewer #1 points 1a and 1b) that addresses this query, which was originally a technical/sequencing depth issue. In short, we now present more saturated pre-enrichment libraries and highlight that these are not used for essential gene predictions (as is standard for Bio-Tradis).

d. The result section (p. 6) summarising the distribution of insertions is misleading, the ~4k insertions if distributed on the whole genome would occur every 17 nt, but here the majority of insertions is only in the first part of the genome, that appears to be injected earlier, as discussed in the next section, please discuss this limitation in a transparent way right away in the results section. In addition, take into account also other explanations from point (1).

We agree that the distribution is uneven throughout the genome and have updated the text. We now present all plots of pre- and post-enriched biological triplicates for phage JS26 and for the post-enriched TnpA and Uvx-TnpA samples for PCH45. These plots enable readers to easily assess the insertion distributions visually across the genome and replicate samples.

2. The claim that Phage Tn-seq works for phages with (hyper)modified genomes is a strong one and very much required for the broad applicability of this method for general phage biology as many phages share modified genomes, hence important for the method. In this case Tn5 inserts randomly and would be also affected by cytosine modifications, unlike Himar1. Unfortunately, there is not much data provided on the LC53 and Bas46 case in the manuscript that would demonstrate efficient transposon insertion, as claimed in the abstract. In Fig. 4B, the Tn+ plaque showed 4-5 magnitudes less PFUs compared to the wt lysate at (-) condition, similar for LC53, this is a deleterious effect for the library, not comparable to J26 and PCH45, please comment on this. The authors do show that insertions can occur, but the efficiency of transposon mutagenesis should be evaluated for LC53 in a similar depth as for the other Prodigiosinella phages, or it must be discussed that it works but with major limitations related to efficiency.

We agree that the application of phage Tn-seq to hypermodified phages will be of utility to the phage community. To support the feasibility and application of Tn*5* transposition into modified phages, we have added pre- and post- enrichment library titres for both LC53 and Bas46 (new **Fig. S4G,H**) alongside plaque PCR confirmation (new **Fig. S4E**) and whole genome-sequencing of individual mutants (new **Fig. S4I**). Transposon insertions were detected in sites containing multiple modified cytosines, suggesting that 5-arabinose-hydroxy-cytosine and 5-arabinose-arabinose-hydroxy-cytosine modifications do not block Tn*5* insertion. Despite being optimised for unmodified phages during our method development, Tn*5* yielded mutants in these modified phages without protocol adjustments. Furthermore, in the pre-print from the Harms lab, phage Tn-seq of T4 (a modified phage related to LC53 and Bas46) was successful, further supporting that modified phages are accessible to transposon mutagenesis. We cannot currently conclude whether transposition efficiency is equivalent between modified and unmodified phages and therefore, have been more cautious with our interpretation and discussion of possible limitations. It is worth noting that diverse phage DNA modifications are known for all bases (A, G, C, T), so Tn*5*, by virtue of accessing any site, should be inherently more applicable to modified phages than Himar/Mariner(restricted to TA).

Clarification of spot titre assays (original **Fig. 4B**): The original spot titre for LC53 and Bas46 compared a tn+ phage isolated from a single Tn+ plaque with a concentrated WT lysate, resulting in different overall titres. Our intention was not to compare absolute titres, but rather to compare the relative ability of WT and tn+ phages to infect +/- LbuCas13. To remove any confusion, we repeated the assays using WT and Tn+ lysates with comparable titres. These results more clearly demonstrate that the Tn+ phages efficiently overcome LbuCas13a targeting (new **Fig. 4F,G**). We have updated the manuscript accordingly.

3. In the here used transposon, the anti-CRISPR gene is not transcribed from a specific promoter but basically by readthrough, that allows for the analysis of directionality, as done, but at the same time also limits the selection via anti-CRISPR proteins towards highly expressed genes, as discussed. This can also lead to challenges related to the interpretation of essentiality. The

data was correlated to proteomics and conservation to validate the outcomes, that matched for the majority of the cases.

a. But, would it be not more straight forward to correlate this also to the mRNA levels, and include this also as a factor for benchmarking essentiality? Transcriptomics has a much bigger dynamic detection range, and it would allow to understand which expression of the anti-CRISPR gene is required for the negation of the CRSIRP-based selection step. mRNA levels could be also correlated to the insertion index. How do the expression data in Suppl. Fig. 2C correlate with the insertion index pre-enrichment? This should be also included in the interpretation of essentiality, here also the nucleus-forming phage data could be of value as there are many more non-essential genes well distributed in the genome.

   Thank you for your suggestion. To clarify, the anti-CRISPR gene is transcribed from a strong constitutive promoter (see point 3b below) encoded on the transposon upstream of the anti-CRISPR gene. Hence, while readthrough from upstream genes may enhance Acr expression, it is not required for expression of the anti-CRISPR gene itself. In agreement, we observe insertions in intergenic regions outside of operons and within genes (gp14) that are not expressed (i.e., no mRNA transcripts were mapped in our earlier work) or expressed at lower levels (see **Fig. 2B,E**)[5]. Regarding the insertion directionality, we provide a further discussion in the manuscript (see Reviewer #1, point 2a) that includes the possible contribution of detrimental RNAP collisions from transposon insertion in opposite transcriptional direction.

   In terms of benchmarking essentiality, we think that expression is not a good proxy for essentiality because many non-essential genes are expressed. We consider our combination of dCas13d knockdown and proteomics of the mature capsids to provide stronger validation for a selection of the predicted essential genes. However, we have re-run correlations of RNA-seq expression level with insertion index (i.e. essentiality) and included the pre-enrichment samples (see new **Fig. S2H**). A moderate positive association was observed post-enrichment ($S = 3602$, $\rho = 0.536$, $p = 8.9 \times 10^{-4}$), but no significant correlation was found pre-enrichment, or when the early genes (gp01-gp14) were excluded (either pre or post-enrichment).

b. In general, it should be also discussed that this bias could be overcome by utilising early phage-derived and strongly expressed promoters for the expression of the anti-CRISPR protein.

   As discussed above, the anti-CRISPR gene is transcribed from a strong constitutive promoter encoded on the transposon upstream of the anti-CRIPR gene. We selected the JS23100 constitutive promoter which is part of the Anderson promoter collection (https://parts.igem.org/Part:BBa_J23100) and has been shown to successfully drive gene expression in several engineered phages[6,7]. While utilising early phage-derived and strongly expressed promoters is possible, it would require identifying promoter expression levels for each phage of interest separately, making the method much less broadly applicable.

c. LbuCas13 with AIcrVIA3 was here introduced with the idea to use it in combination with the previous LseCas13 AcrVIA1 system. It would be consequently also important to show this, e.g. by delivery of a second reporter in any of the here used phages, e.g. to PCH45::Tn5-mEGFP-AcrVIA1 insert an AIacr via transposons or vice versa, or in another species.

   Thank you for the suggestion. We now designed an LbuCas13a spacer targeting JS26 and used the Tn5-AIcrVIA3 plasmid followed by LbuCas13-mediated counter-selection to deliver an AIcrVIA3-encoding transposon into a JS26::Tn5-mEGFP-AcrVIA1 mutant, successfully creating a JS26::Tn5-mEGFP-AcrVIA1/Tn5-AIcrVIA3 double mutant. Insertion was confirmed *via* plaque PCR and EOP. The data was added to the results and **Fig. 4D, E**.

Minor comments

1. Curiously, in Suppl. Fig. 4B, the GFP is not uniformly distributed as in Fig. 4A and it would be expected for a single protein. Could a read-trough in frame be introduced by transposon insertion that results in a phage-GFP fusion protein? Was there a stop codon inserted in each frame before the GFP gene? This would be required for gene delivery to phage genomes. For the transposon it would not make much of a difference, but it would be still better to have it not fused by coincidence to another protein that could introduce bias. As a remark, GFP can also aggregate at stress. Please clarify on the plasmid/transposon design related to this point, also in the method section.

   We have clarified the transposon design in the method section. All three reading frames upstream of the mEGFP gene encode stop codons, preventing accidental fusion with phage proteins. In addition, we have whole genome sequenced of both mutants used in this manuscript, confirming the exact insertion site. Regarding the GFP distribution in JS26 infected cells, we suspect the nonuniform distribution was caused by technical issues. We have repeated the experiment and updated **Fig. S4B**, showing a more uniform GFP distribution.

2. To show also applicability of Phage Tn-seq for the nucleus-forming phage, the transposase was fused to a phage protein for import into the phage nucleus and the authors claim that this boosted the efficiency. As it is anyway interesting why it worked out at all without fusion to recA, this is an important point to understand nucleus-forming phage biology.

   a. Suppl. Fig. 3G shows that this is only true for 2/3 replicates, and B showed in general less phage titre, please comment on this, and extend the sample size. This will solidify if the requirement for import into the phage nucleus for efficient mutation is true.

      We have since repeated the experiment in response to major comment 1b of reviewer #1 and also included the PFU/ml data for all libraries prior to enrichment in an updated **Fig. S3G** and **Table S2**. These data show a clear reproducible improvement in phage titres post-enrichment on the *uvsX*::*tnpA* fusion (**Fig. S3G**) and more informatively, an increased insertion density when nuclear import of the transposase occurs (**Table S2**).

   b. In addition, these lysates were already enriched but why is there then still in 2/3 cases ~2 log difference in Suppl. Fig. 3G. Any library after enrichment should reach similar titres as the mutagenized phages will replicate. Was this checked on a lysate prior enrichment?

      We have since repeated the experiment in response to major comment 1b of reviewer #1 and added the phage titres for all libraries prior to enrichment (**Fig. S3G**). Regarding the difference in phage titres of the libraries post-enrichment when plated on the WT *vs.* when plated on the selection: potentially, there are still some WT phages present in the library, extending the enrichment step using new culture will most likely result in equal titres. It is also possible that some bacterial surface resistance to phages can emerge during outgrowth, which would stall further Cas13-mediated enrichment of transposon mutants. Overall, this did not impact our ability to generate a sufficient density of insertions for essentiality predictions.

   c. Suppl. Fig. 3E shows the pre-enrichment transposon insertions for the tnpA experiment (l. 322). But due to low saturation, tnpA was fused to recA (l. 327 ff., Fig. 3D), but the pre-enrichment library is not shown anywhere, please show the pre-enrichment library for Fig. 3D in the Suppl. Fig. 3.

      See our responses to 2a and 2b above.

3. Please discuss if there are any limitations related to phages that degrade the host DNA and henceforth the transposon.

   Host DNA degradation upon infection occurs for a variety of phages. For example, *E. coli* phage T4 can degrade bacterial DNA within a matter of minutes[8–11]. Since LC53 and Bas46 are T4-like phages, they are likely to act similarly[12,13], but we were still able to generate transposon

mutants. In agreement, in the other phage Tn-seq pre-prints using the mariner transposon, mutants were readily generated in phages T4 and in phiKZ, which are known to degrade host DNA. We think that transposase expression prior to phage infection will lead to the formation of transpososomes (i.e. Transposase-DNA complexes) that are proficient for mutagenesis prior to host DNA degradation by phage nucleases[14].

4. Please discuss if there are any limitation related to phages that encode anti-CRISPR proteins for the selection step.

   If the phage of interest naturally encodes the Acr gene used for counterselection, then the system will not work, since selection for transposon mutants will be unsuccessful. However, as discussed in the manuscript, alternative systems are available, that could be used in such a case – such as our demonstration of the orthogonal nature of LbuCas13 and LseCas13 and their anti-CRISPRs.

5. As a general remark, it would boost the accessibility of this method to include quantitative PCR readouts for the general estimation of insertion saturation and for specific targeted loci to test for their depletion without the requirement to go for sequencing.

   Unfortunately, we don't think quantitative PCR would be well suited to estimate insertion saturation for this type of mutant library. While amplification of the transposon could be compared to amplification of a housekeeping gene, the method will not be able to distinguish between insertions in the same loci vs insertions in different loci.

   An alternative, lost-cost approach to confirm unique insertions in the phage mutagenesis library is to perform arbitrary PCR on individual plaques following counterselection (using a transposon-specific primer and degenerate primer sets), followed by Sanger sequencing of resulting amplicons. This would give confidence that the phage pool has insertions in distinct genomic locations prior to deep sequencing.

   For confirmation of insertions into specific loci, a simple PCR (junction PCR) using a primer binding to the genome region of interest in combination with a primer binding to transposon would be sufficient to verify insertions.

6. l. 18: one CRISPR too much or rephrase it to split the acr and the CRISPR.

   The additional "CRISPR" has been removed.

7. l. 120: Reference 22 did not make use of Acrs.

   Reference 22 has been added to reference the diverse use of Cas13a, which is also mentioned in the associated sentence.

8. l. 127: the "tightly inducible" araBAD promoter is in mine and other experience not that tight, this is a bit misleading, please rephrase, and make aware about potential issues and ways out in case of toxicity.

   The wording has been adjusted to remove "tightly". Regarding toxicity, please also see our response to minor comment 3 of Reviewer #1.

9. l. 131: Plasmids are not available on Addgene for now, please list the identifiers in the manuscript. Very much appreciated to share the plasmids on Addgene!

   Plasmids pPF4213 (#254224), pPF4354 (#254225), pPF4481 (#254226), pPF4485 (#254227) and pPF3929 (#254228) are now available on Addgene and we have included their numbers.

10. Suppl. Fig. 3 legend, E is missing.

    "E" has been added.

11. l. 318: This sequenced library, was it harvested before or after enrichment? This is critical to be comparable to the next section, please clarify in the text.

    The enriched mutant pool was used for sequencing. To clarify, we adjusted the sentence to: *"Following enrichment of the transposon mutant lysates, we deep-sequenced the transposon junctions in the enriched PCH45 transposon mutant pools…".*

12. l. 324: The fact that transposon mutagenesis works without specific localization to the phage nucleus is more than notable. As it is also described that plasmid DNA can recombine with the phage genome, could it be that the transposase/transposon move inside via DNA? This would be worth mentioning in the discussion.

    In theory, the transpososome may be sporadically encapsulated in the forming phage nucleus, resulting in low-frequency transposon insertion into the phage genome. We have added this discussion point to the results section.

13. The legend of Fig. 4 contains still elements that are moved to Suppl. Fig. 4, please correct this and update the figure.

    Fig. 4 and **Fig. S4** and their legends have been updated.

Reviewer #3 (Remarks to the Author):

All of my comments have been integrated into the other reviewer's submission.

References

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Responses to reviewer comments

Please see our responses below in blue text.

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

Many thanks to the authors for addressing our comments in a comprehensive manner and congratulations on developing this powerful technology. We have no further comments, only a few notes on typos:

1. The caption for Figure 2 does not line up with the panels, it looks like the caption for Figure 2C should refer to panel E, the caption for 2D to panel C, and the caption for 2E to panel D. In addition, there is a reference to Fig. S2E, but it should likely reference Fig. S2F.
Thanks for spotting this error, which we have now corrected.

2. Line 437: Missing second ")" after "(gp162)".
Thanks for spotting this error, which we have now corrected. Please note that this text is now present in Supplementary Note 2 rather than the main manuscript.

3. Line 641: "fundament" should read "fundamental".
This error has been removed.

4. Line 976: "." after "NCBI_Conserved_domains" should be a ",".
Thanks for spotting this error, which we have now corrected.

5. References 18 and 31 show up in the list as "(!!! INVALID CITATION !!!)".
Referencing has been fixed. This also included updating bioRxiv articles that are now published.

6. We appreciated this sentence in the author response: "Although we cannot rule out possible polar effects in some locations, insertions do occur upstream of essential genes in operons." This is a thoughtful assessment, and we think there would be value to including a sentence that reflects both sides of this assessment in the discussion. That said, the authors should feel free to take or leave this.
Due to the need to reduce the manuscript by almost 40%, we do not consider that there is space to include this.

Reviewer #2 (Remarks to the Author):

The authors addressed all my points, no further requests.

Minor suggestions:
In addition to Reviewer #1 point 2: A Tn5 transposon-induced anti-sense transcript expression into an essential gene could lead to silencing by RNA-RNA duplex formation and RNase III-induced degradation and apparent essentiality for neighboring genes, optionally to be put up in the discussion, as there was no terminator in the transposon.

Thanks for this interesting idea. However, due to the need to reduce length and the speculative nature of this possible mechanism, we have opted to not include this in the manuscript.

Point 3a,b: I do agree that a strong promoter shares benefits, it would be still good to mention that this should match with the RNAP selectivity, e.g. in case of nucleus-forming phages this may cause issues, as phage RNAP is used with specific sigma factors and not the general host RNAPs that are excluded by the nucleus. In essence, I still think, that read-through has a high contribution here that could be mentioned in the discussion.
This is a good point, but less useful as a general method for Phage Tn-seq. We have added the following sentence to the relevant position in the methods, due to the length restrictions in the main text"
"Although a constitutive promoter was used in this study, our results indicate that phage transcriptional read-through also contributes to Acr expression. In systems requiring phage-specific transcriptional control, incorporation of phage promoter elements may therefore be advantageous."

A statement of caution related to general read coverage and insertion bias would be helpful.
We have added the following sentence to the relevant position in the methods, due to the length restrictions in the main text"
"Essentiality analyses were performed only on enriched libraries with sufficient insertion density. We recommend assessing library saturation, sequencing coverage and insertion distributions when applying this approach to additional phages."