nature portfolio

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: Widespread stop-codon recoding in bacteriophages may regulate translation of lytic genes
Corresponding author name(s): Jillian Banfield

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Jill,

Thank you for your patience while your manuscript "Stop codon recoding is widespread in diverse phage lineages and has the potential to regulate translation of late stage and lytic genes" was under peer-review at Nature Microbiology. Please first accept my sincere apologies for the delay in getting back to you with a decision. As I mentioned previously, one of the referees was substantially delayed in returning their comments to us, which really delayed the process. To make matters worse, that reviewer has recently become unresponsive to our chasers, so we were unable to secure that final report. Nonetheless, as the expertise from that reviewer overlaps with those from one of the other referees, we feel confident that all technical aspects of the paper have been adequately assessed by our referees, so decided to make a decision at this stage even though the paper has only been seen by 2 referees, whose expertise and comments you will find at the end of this email. As you will see from their comments, although the referees find your work of interest, they have also raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, you will see that most of the points brought up by referee #1 are related to the need to provide a bit more detailed information on how some of the analyses were performed; expand the reporting of the results of some of those analyses (including more SI tables with the underlying data); and paying more attention to viral nomenclature. Referee #2 provides several suggestions aimed at further strengthening the case for recoding having an important functional role in these phages, including a more detailed analysis of the presence of suppressor tRNAs in AC phage genomes; potentially looking for DNA/RNA modifying enzymes; and exploring the evolutionary relationship between AC genomes with vs without suppressor tRNAs. The referee also suggests some streamlining of the main messages; and that you expand the discussion around other potential roles of recoding and its roles in the microbiome. The rest of the comments made by the referees seem clear and straightforward to address.

Should further experimental data and text modifications 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, so 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

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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

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* 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.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
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Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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Please use the link below to submit a revised paper:

{redacted}

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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, and please let me know if you have any questions.

With best regards,

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Reviewer Expertise:

Referee #1: phage; bioinformatics
Referee #2: recoding

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Reviewer Comments:

Reviewer #1 (Remarks to the Author):

"Stop codon recoding is widespread in diverse phage lineages and has the potential to regulate translation of late stage and lytic genes" by Borges et al is an exciting, well written story. I have only few comments.

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L27: Please check whether crass and lak are really virulent phages. For example the isolated strain crass001 displayed a pseudolysogenic phenotype.
L75-77, L93: "people predicted to eat a westernized diet" it is unclear why or how people's diet was predicted, while this information should be known.
L130: "Some of these phages have Candidatus Absconditabacteria predicted as the host" It would be helpful to refer to a supplementary file/table with information about all the 9322 identified AC contigs, such as the predicted host (method), other information indicated in Figure 2A, circularity, completeness estimate, and importantly also their sequence identifier in the public database so that one can retrieve the sequence.
L137-139: "we chose to name the six new clades of TAG and TGA recoded phages after other gemstones (Garnet, Amethyst, Jade, Sapphire, Agate, Topaz)" It should be stated that these names are given within the scope of this paper and are not intended as taxonomic names. It would be very helpful if the authors would propose these clades as official viral taxa through taxonomy proposals by contacting ICTV.
L235-236: "DNA replication machinery represented as a yellow bar and structural and lysis genes with a pink bar." It is unclear what this annotation is based on, the methods are very limited and no details are provided (L627). It would be helpful to add results of the gene annotations in a supplementary table, at least for the genomes that are shown/discussed in the manuscript. It would also be informative to see e.g. how many of the tryptophan residues were encoded by TGA codons in each gene. This information is critical to assess the significance of the statement that "accumulation of TGA codons in structural and lysis genes, but not the nearby DNA replication machinery" (L203 and L237).
L236: "TGA stop codons have arisen in structural and lysis genes (individual recoded genes below in green) while DNA replication machinery has not accumulated stop codons." I think that calling these "stop codons" may not make sense; as far as I understand it the TGA are not stop codons but code for tryptophan in the c4 code. Consider rephrasing "stop codon" to "TGA or TAG codon" or maybe "recoded stop codon", also in L248, L251 and potentially elsewhere. In L375 it is OK.
L290 "A strongly recoded transmembrane domain protein" Based on P=4.64e-2 maybe remove "strongly"?
L317 "Origins and termini were identified based on GC skew patterns" Please show details of these results, else it is difficult to judge the validity of the inferred Ori sites.
L324: please state the hypothesis that was tested with the BH test.
L337, L343, L346: S8A should be S7. There is only A and no panel B. Etc.
L351: "We also identified circular free phage genomes in our dataset" To clarify, please state where these were identified/that they were ideitified in different samples.
L712: "the integrase gene families strongly avoided in-frame stop codons relative to the rate at which they used standard codons" I would expect stop codons to be avoided in-frame in any ORF, so this is not surprising. I thought the test was to be between in-frame stop codons in integrase genes versus other genes.

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Reviewer #2 (Remarks to the Author):

Borges et al. present a bioinformatics analysis of stop codon recoding in bacteriophages of the human

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and animal microbiome, and suggest that reassignment of stop codons may serve as a dynamic regulatory mechanism to control the phage life cycle. The presented work is clever and original as well as of quality and interest.

The manuscript is generally well written but would benefit from another round of revision to clarify its narrative and main message. Moreover, while some level of speculation on biological mechanisms is unavoidable in such large-scale computational studies, few additional analyses within immediate reach may clarify some of that speculation; the authors may want to consider the following comments that aim to help them improve on their work.

1) The central tenet of this work is the identification of stop codon recoding based on predicted coding density in phage genomes. Please expand on your illustration in support of this argument to make the power of this idea more immediately accessible to the reader. For instance, Figs 1D-I clearly show that the AC genomes have lower coding densities with the standard code than the rest of the genomes. In the methods section you state that a minimum increase in coding density of 5-10% was required to define genomes as AC. Can you show, in Fig. 1 or a supplementary figure, how the data points for the AC genomes in Fig. 1D-I shift upon your reassignment of genetic codes? Moreover, the steps described in the methods (lines 616-625) are sensible and thorough; it could be nice to graph some of these analyses in validating supplementary figures as the whole paper is built upon this definition.

2) The identification of suppressor tRNAs in 35-40% of the AC genomes is substantial, offers a direct mechanistic explanation, but leaves as many questions as answers. The authors state themselves that this number may be much higher than can be detected with the established state-of-the-art algorithm tRNAscan, which was primarily developed for eukaryotic organisms. Clarification of the number of AC phage genomes with suppressor tRNAs could much strengthen this work.
Specifically, you here only have < 500 AC genomes with complete or near-complete genome sequences that are generally small, and each with very high conding density. I.e. you have only so many sequences left that could potentially encode a suppressor tRNA. While there may be noncanonical structures, the tRNA stem-loop structure is generally ultra-conserved and somewhat constrained by the ribosome. RNA structure prediction is readily accessible and you could e.g. try to compare RNA structures of truncations of the remaining sequences by structure-based sequence alignments or structure superposition with the stem-loop region of tRNAs. Or, conversely, can you in fact exclude for some of these genomes that they encode suppressor tRNAs? This would also be very important to know.
Alternatively, are the identified suppressor tRNAs preferentially found in complete genomes while there may be biases in resolving tRNA sequences in genome sequencing and assembly that could explain some of the 'missing' cases in the 'nearly' complete genomes? This is for example the case with ribosomal RNA that are often missing from genome assemblies due to their repetitive elements. Or, do the phages predicted as AC without suppressor tRNAs exhibit lower increases in coding density upon genetic code reassignment, thus are potentially less clear-cut candidates for AC genomes?
If possible please try to explore a little deeper the presence of suppressor tRNAs in AC phage genomes as this would really strengthen your results.

3) The switch to AC translation as means to dynamically regulate the biosynthesis of lysis genes is striking. However, this would imply that initially these genes are translated with the 'wrong' genetic

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code and produce aberrant protein waste, thus a burden on the host. Why could it be a beneficial strategy to dyamically control translation and biosynthesis of these genes through suppressor tRNAs rather than mechanisms that would initially prevent their translation alltogether, e.g. sequestering of RNA through RNA-binding proteins or transcriptional control? I would think that DNA/RNA modifying enzymes etc. could as easily be included in the phage genomes as the suppressor tRNAs. Could it be energetically or otherwise beneficial to initially mistranslate these genes? Or could there be wider consquences on the host organism wherein the switch to AC also selectively inhibits host genes that become nonfunctional once the suppressor tRNAs are present that in turn benefit the phage life cycle?

4) If there is anything known about putative functional consequences or advantages of recoding in the examples mentioned in the introduction (lines 37-43), please consider making brief mention of this in the introduction at the very beginning for context.

5) The authors speculate that recoding could act as a sensor for the presence of coinfecting or superinfecting phages. Extending this idea in context that there is usually a population of phages in the microbiome rather than individual phages, could the phages with suppressor tRNAs play a dominant role in that population and coordinate interdependencies between different phages through sharing of suppressor tRNAs? This would become a relevant question if indeed not all AC phages bring their own suppressor tRNA.

6) What is the evolutionary relationship between AC genomes with and without suppressor tRNAs? Do the AC genomes without suppressor tRNAs partition distinctively in a way that could let assume some hierarchy between phages within a population? Conversely, if the number of phages with suppressor tRNAs is finally much higher, such an analysis could also show evidence of coevolution of recoding and the emergence of suppressor tRNAs.

7) Please overall revise the presentation. I found the initial narrative a little confusing and the intention of the work was most clear to me after reading the discussion. You should be able to address this with minimal clarifications in abstract and introduction, for example by making mention of known functions of recoding, if known. Please define or describe suppressor tRNAs at first mention. Some of the figures may benefit from increasing the font size of the axes labels and adding labels. For example, Figure 3B is difficult to read, even together with the legend.

Author Rebuttal to Initial comments

Response to reviewers:

We thank the reviewers for their enthusiasm towards our work, as well as for the critical feedback which has improved the manuscript. We are aware of the time it takes to give thorough reviews of manuscripts, and are very appreciative of the thoughtful comments that were provided.

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Responses to Reviewer 1:

"Stop codon recoding is widespread in diverse phage lineages and has the potential to regulate translation of late stage and lytic genes" by Borges et al is an exciting, well written story. I have only few comments.

We appreciate the enthusiasm for our work, and thank the reviewer for their feedback.

L27: Please check whether crass and lak are really virulent phages. For example the isolated strain crass001 displayed a pseudolysogenic phenotype.

This is a good point, we were assuming a virulent phenotype based on lack of integrases and lack of integrated genomes, but this approach does not account for pseudolysogenic lifestyles. We have chosen to remove the instances where we refer to phages as "virulent" based on the aforementioned characteristics, as an obligate lytic phage lifestyle is not central to any of our conclusions.

L75-77, L93: "people predicted to eat a westernized diet" it is unclear why or how people's diet was predicted, while this information should be known.

We have added clarification in the main text that diet type was provided by the studies that generated the initial metagenomic datasets, or was inferred based on geographic location. For example, we inferred that the diet of Pennsylvania, USA residents would be similarly "westernized" as the diets of Oklahoma, USA residents.

L130: "Some of these phages have Candidatus Absconditabacteria predicted as the host" It would be helpful to refer to a supplementary file/table with information about all the 9322 identified AC contigs, such as the predicted host (method), other information indicated in Figure 2A, circularity, completeness estimate, and importantly also their sequence identifier in the public database so that one can retrieve the sequence.

Thank you for the suggestions. Supplementary Table 1 has phage contig name, genetic code, genome length, gc content, clade, host/host method, circularity, completeness, and a few other basic data for all the alternatively coded phages as well as their standard code relatives. These genomes are currently under submission to the ENA, and the final spreadsheet will be updated

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with the accession code for each genome. The genome and proteome files for each of these phages are also immediately available as supplementary datasets Supplementary Data File 1 and Supplementary Data File 2, respectively.

L137-139: "we chose to name the six new clades of TAG and TGA recoded phages after other gemstones (Garnet, Amethyst, Jade, Sapphire, Agate, Topaz)" It should be stated that these names are given within the scope of this paper and are not intended as taxonomic names. It

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would be very helpful if the authors would propose these clades as official viral taxa through taxonomy proposals by contacting ICTV.

We have added the suggested clarification in the text, as we have not begun the process of proposing these phages clades as official viral taxa.

L235-236: "DNA replication machinery represented as a yellow bar and structural and lysis genes with a pink bar." It is unclear what this annotation is based on, the methods are very limited and no details are provided (L627). It would be helpful to add results of the gene annotations in a supplementary table, at least for the genomes that are shown/discussed in the manuscript. It would also be informative to see e.g. how many of the tryptophan residues were encoded by TGA codons in each gene. This information is critical to assess the significance of the statement that "accumulation of TGA codons in structural and lysis genes, but not the nearby DNA replication machinery" (L203 and L237).

We have added the suggested gene tables for all phage genomes that we show genome maps for in the main text, or the supplementary text.

L236: "TGA stop codons have arisen in structural and lysis genes (individual recoded genes below in green) while DNA replication machinery has not accumulated stop codons." I think that calling these "stop codons" may not make sense; as far as I understand it the TGA are not stop codons but code for tryptophan in the c4 code. Consider rephrasing "stop codon" to "TGA or TAG codon" or maybe "recoded stop codon", also in L248, L251 and potentially elsewhere. In L375 it is OK.

Thank you for pointing this out, we agree it is confusing and have changed all instances of this language to say “recoded stop codon”.

L290 "A strongly recoded transmembrane domain protein" Based on P=4.64e-2 maybe remove "strongly"?

We have changed the language to “a preferentially recoded transmembrane domain protein”, as the goal here was to communicate that this gene family is biased towards use of the TGA codon to encode Tryptophan, relative to the standard TGG Tryptophan encoding.

L317 "Origins and termini were identified based on GC skew patterns" Please show details of these results, else it is difficult to judge the validity of the inferred Ori sites.

We have added the suggested cumulative GC skew plots in the supplementary data (Supplementary Figure 2A-I for all the phages that we show full genome maps for.

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L324: please state the hypothesis that was tested with the BH test.

We have clarified this, adding a sentence explaining that we were " measuring rates of TAG use relative to standard code encoding of glutamine (C, TAG →Q recoded phage in Fig. 4C) or TGA use relative to the standard code encoding of tryptophan (TGA →W recoded phage in Fig. 4D)".

L337, L343, L346: S8A should be S7. There is only A and no panel B. Etc.

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Noted and fixed.

L351: "We also identified circular free phage genomes in our dataset" To clarify, please state where these were identified/that they were identified in different samples.

We have clarified that these circular free phage genomes come from related baboon samples.

L712: "the integrase gene families strongly avoided in-frame stop codons relative to the rate at which they used standard codons" I would expect stop codons to be avoided in-frame in any ORF, so this is not surprising. I thought the test was to be between in-frame stop codons in integrase genes versus other genes.

We apologize for the inaccurate phrasing used here, this was our mistake. To clarify - In this analysis, we used the same statistical tests as we did with the Agate and crAss phages where we identified preferentially recoded genes. In this scenario however, we looked for gene families that were de-enriched for recoded stop codons. We have added the following language to the manuscript: “This statistical test was used to analyze rates of TAG use relative to standard code encoding of glutamine (TAG →Q recoded phage) or TGA use relative to the standard code encoding of tryptophan (TGA →W recoded phage)”

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Reviewer #2 (Remarks to the Author):

Borges et al. present a bioinformatics analysis of stop codon recoding in bacteriophages of the human and animal microbiome, and suggest that reassignment of stop codons may serve as a dynamic regulatory mechanism to control the phage life cycle. The presented work is clever and original as well as of quality and interest.

The manuscript is generally well written but would benefit from another round of revision to clarify its narrative and main message. Moreover, while some level of speculation on biological mechanisms is unavoidable in such large-scale computational studies, few additional analyses within immediate reach may clarify some of that speculation; the authors may want to consider the following comments that aim to help them improve on their work.

We thank the reviewer for their kind appraisal of our work, and for their thoughtful suggestions regarding tRNA biology.

1) The central tenet of this work is the identification of stop codon recoding based on predicted coding density in phage genomes. Please expand on your illustration in support of this argument to make the

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power of this idea more immediately accessible to the reader. For instance, Figs 1D-I clearly show that the AC genomes have lower coding densities with the standard code than the rest of the genomes. In the methods section you state that a minimum increase in coding density of 5-10% was required to define genomes as AC. Can you show, in Fig. 1 or a supplementary figure, how the data points for the AC genomes in Fig. 1D-I shift upon your reassignment of genetic codes? Moreover, the steps described in the methods (lines 616- 625) are sensible and thorough; it could be nice to graph some of these analyses in validating supplementary figures as the whole paper is built upon this definition.

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We thank the reviewer for the suggestions, and we have addressed this shortcoming in three ways. First, we added a few more lines in the main text to explain the logic of using coding density as an indicator of alternative coding. Next, we have made a flow chart in our supplementary figures (Supplementary Figure 1A) to show our analytical pipeline alongside two example alternatively coded genomes with coding density calculated in standard code, code 4, and code 15 (Supplementary Figure 1B-C). Third, we have generated the suggested figures where we show the data points from Fig. 1D-I shifting up when the coding density is calculated with the alternative genetic code (Extended Data Figure 2).

2) The identification of suppressor tRNAs in 35-40% of the AC genomes is substantial, offers a direct mechanistic explanation, but leaves as many questions as answers. The authors state themselves that this number may be much higher than can be detected with the established state-of-the-art algorithm tRNAscan, which was primarily developed for eukaryotic organisms. Clarification of the number of AC phage genomes with suppressor tRNAs could much strengthen this work.

Specifically, you here only have < 500 AC genomes with complete or near-complete genome sequences that are generally small, and each with very high conding density. I.e. you have only so many sequences left that could potentially encode a suppressor tRNA. While there may be noncanonical structures, the tRNA stem-loop structure is generally ultra-conserved and somewhat constrained by the ribosome. RNA structure prediction is readily accessible and you could e.g. try to compare RNA structures of truncations of the remaining sequences by structure-based sequence alignments or structure superposition with the stem-loop region of tRNAs. Or, conversely, can you in fact exclude for some of these genomes that they encode suppressor tRNAs? This would also be very important to know.

Alternatively, are the identified suppressor tRNAs preferentially found in complete genomes while there may be biases in resolving tRNA sequences in genome sequencing and assembly that could explain some of the 'missing' cases in the 'nearly' complete genomes? This is for example the case with ribosomal RNA that are often missing from genome assemblies due to their repetitive elements. Or, do the phages predicted as AC without suppressor tRNAs exhibit lower increases in coding density upon genetic code reassignment, thus are potentially less clear-cut candidates for AC genomes?
If possible please try to explore a little deeper the presence of suppressor tRNAs in AC phage genomes as this would really strengthen your results.

We thank the reviewer for bringing this issue of missing suppressor tRNAs to the forefront as it prompted us to do some important analyses:

First, we tested to see if incomplete genomes were driving the low number of identified suppressor tRNAs. However, we found that the rate of suppressor tRNA identification did not substantially change when we considered only the circularized phage genomes. 42% of TAG

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recoded circular phages had TAG suppressors (increased from 40%) and 38% of TGA recoded circular phages had TGA suppressors (increased from 35%).

Next, we pursued the evolutionary angle suggested by the reviewer in comment 6. When we mapped the occurrence of suppressor tRNAs onto our phylogenetic tree of alternatively coded phages we saw a very clear and interesting pattern. Here, phages with large genomes encoded suppressor tRNAs at high rates, while suppressor tRNAs were nearly absent from small genome phages (See our revised Figure 2A).

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A strong positive association between tRNA gene number and genome size has been observed before, so our finding that small phages also lack suppressor tRNAs is consistent with that literature. However, we were very surprised to see whole families of alternatively coded phages were nearly devoid of these important genes.

We next followed the reviewer's suggestion to search the intergenic regions for structured ncRNAs. We focused this analysis on 240 genomes from the Topaz phage clade, due to the high number of AC phage genomes without predicted tRNAs. We used CMfinder to find structured RNAs, and combined these results with Infernal in an iterative framework to expand the possible identified ncRNAs with additional sequences. This is an unpublished pipeline developed in our lab by Dr. Petar Penev (a ncRNA expert, now an author on this paper) to identify non-coding RNAs, and we have validated that it can be used to discover tRNAs. However, we were unable to discover more tRNA-like sequences in Topaz phage genomes with this approach.

We also approached the question from the other side - that is to take the few tRNAs we do find in Topaz clades with tRNAscanSE, and construct covariation models of just them, then use these Topaz-specific models to search for tRNAs within the Topaz genomes. This expanded the number of tRNAs by a few (9) and none of the newly discovered were suppressors, they were mostly undetermined and Ser.

Though it is very difficult to prove a negative and conclusively demonstrate that most small-genome AC phages do not encode suppressor tRNAs, we feel that is the most fair interpretation of the data we have right now. We speculate that this finding may reflect a hierarchical relationship and/or parasitic relationship between large and small phages, and are excited for future research to more systematically interrogate our proposed models.

3) The switch to AC translation as means to dynamically regulate the biosynthesis of lysis genes is striking. However, this would imply that initially these genes are translated with the 'wrong' genetic code and produce aberrant protein waste, thus a burden on the host. Why could it be a beneficial strategy to dyamically control translation and biosynthesis of these genes through suppressor tRNAs rather than mechanisms that would initially prevent their translation alltogether, e.g. sequestering of RNA through RNA-binding proteins or transcriptional control? I would think that DNA/RNA modifying enzymes etc. could as easily be included in the phage genomes as the suppressor tRNAs. Could it be energetically or otherwise beneficial to initially mistranslate these genes? Or could there be wider consquences on the host organism wherein the switch to AC also selectively inhibits host genes that become nonfunctional once the suppressor tRNAs are present that in turn benefit the phage life cycle?

These are all really interesting ideas, and future work will be needed to address them to the full

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extent that they deserve. Here are our thoughts on the matter so far: While it is possible that these phages only use translational control through alternative coding to regulate protein production, we consider it more likely that these phages do use transcriptional controls as well. In this scenario, stop codon recoding provides an extra level of regulation on top of existing RNA expression patterns. We think this extra level of translational control would be most relevant when in scenarios where the phage would undergo transcriptional de-regulation, potentially due to host interference or co-infecting phages. We have noted both of these scenarios in the discussion section, as we consider them to be the most likely players here.

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There is also a possibility that the truncated gene products could be functionally important in some way, i.e. this could be a way to expand the phage proteome. Proteome expansion via recoding is seen in yeast *Candida albicans*, which we now reference in the introduction. We don't have any evidence to support such a model applying in our system, though we are working to address these questions in future work. The question about the impact on the host is also unexplored. Interestingly, suppressor tRNAs have been used in genetics for a long time and surprisingly don't have extreme negative effects when expressed in *E. coli* host bacteria (https://journals.asm.org/doi/10.1128/JB.186.20.6714-6720.2004). If such antagonism is occuring, it is likely very host/strain specific and is difficult to predict without isolation of phage-host pairs.

4) If there is anything known about putative functional consequences or advantages of recoding in the examples mentioned in the introduction (lines 37-43), please consider making brief mention of this in the introduction at the very beginning forcontext.

We appreciate the suggestion, and have added a section to the introduction to discuss functional consequences of recoding.

5) The authors speculate that recoding could act as a sensor for the presence of coinfecting or superinfecting phages. Extending this idea in context that there is usually a population of phages in the microbiome rather than individual phages, could the phages with suppressor tRNAs play a dominant role in that population and coordinate interdependencies between different phages through sharing of suppressor tRNAs? This would become a relevant question if indeed not all AC phages bring their own suppressortRNA.

We are very interested in these ideas, and have added a section in the discussion to discuss the relationship between suppressor-encoding phages and phages that lack suppressor tRNAs (more on that below).

6) What is the evolutionary relationship between AC genomes with and without suppressor tRNAs? Do the AC genomes without suppressor tRNAs partition distinctively in a way that could let assume some hierarchy between phages within a population? Conversely, if the number of phages with suppressor tRNAs is finally much higher, such an analysis could also show evidence of coevolution of recoding and the emergence of suppressortRNAs.

Our new findings that large phages tend to encode suppressor tRNAs, but small phages lack them is very intriguing. We speculate that there could indeed be a hierarchical relationship between alternatively coded large phages that encode suppressor tRNAs and alternatively

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coded small phages that lack them. We have added a section in the discussion where we propose that small phages may be “piggy-backing” on large phages for translational resources. This model of suppressor tRNA sharing could also potentially allow any co infecting phages to synchronize their infection cycles, which would likely be beneficial to all phages.

7) Please overall revise the presentation. I found the initial narrative a little confusing and the intention of the work was most clear to me after reading the discussion. You should be able to address this with minimal clarifications in abstract and introduction, for example by making mention of known functions of recoding, if known. Please define or describe suppressor tRNAs at first mention. Some of the figures may benefit from increasing the font size of the axes labels and adding labels. For example, Figure 3B is difficult to read, even together with the legend.

Thank you for the suggestions. 1) We have re-written our abstract and introduction and incorporated this feedback. We now have an intro paragraph that discusses the known benefits of recoding and alternative genetic codes. 2) We have broken out a results section for “Mechanisms of phage-induced code change” where among other changes we more clearly define suppressor tRNAs. 3) We have increased figure font sizes and improving our axis labels
4) We have moved figure 3B to the supplement and increased its size as we agree it is hard to read/interpret, especially at a small size. In its place in the main text, we show plots of GC content and stop codon use across standard code phages, as we feel those get across our point about codon capture better anyway.

Decision Letter, first revision:

Dear Dr. Banfield,

Thank you for submitting your revised manuscript "Stop codon recoding is widespread in diverse phage lineages and has the potential to regulate translation of late stage and lytic genes" (NMICROBIOL-21092194A). I'll be your handling editor for these final steps, because {redacted} has now left the journal.

It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore 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.

Please email me a copy of the file in an editable format (Microsoft Word preferably)-- we cannot proceed with PDFs at this stage.

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

{redacted}

Reviewer #1 (Remarks to the Author):

Thank you for the edits, I am satisfied. This remains a very exciting study.

Reviewer #2 (Remarks to the Author):

I am fully satisfied with the authors' revisions and responses, and support publication without further delay.

Decision Letter, final checks:

Dear Dr. Banfield,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "Stop codon recoding is widespread in diverse phage lineages and has the potential to regulate translation of late stage and lytic genes" (NMICROBIOL-21092194A). Please accept my sincere apologies for the delay in sending this guidance. I've taken a very careful read of the manuscript and edited accordingly. The main area where I think a little work is needed is in the Introduction where the scene setting needs to mention the different genetic code designations that are used in the results. I hope my comments are helpful, they are well meant and if they reveal any lack of understanding on my behalf, I'm very sorry! Its all done using track changes but if anything is unclear do let me know.

Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

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In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Stop codon recoding is widespread in diverse phage lineages and has the potential to regulate translation of late stage and lytic genes". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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{redacted}
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Reviewer #1:
Remarks to the Author:
Thank you for the edits, I am satisfied. This remains a very exciting study.

Reviewer #2:
Remarks to the Author:
I am fully satisfied with the authors’ revisions and responses, and support publication without further delay.

Final Decision Letter:

Dear Professor Banfield,

I am pleased to accept your Analysis "Widespread stop-codon recoding in bacteriophages may regulate translation of lytic genes" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

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