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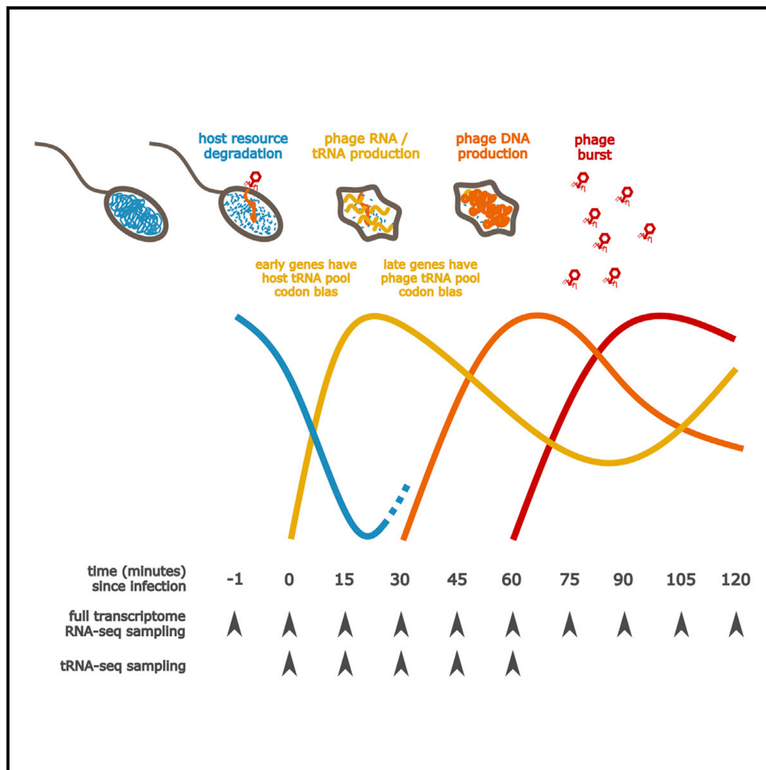
Article

Degradation of host translational machinery drives tRNA acquisition in viruses

Graphical abstract



Highlights

- We ask what selective pressures drive tRNA carriage in a T4-like Vibriophage
- Phage tRNAs are expressed at levels adapted to phage codon usage in late genes
- Random acquisition of the diverse array of 18 phage tRNAs observed is unlikely
- The phage has a dynamically adapted codon usage strategy

Authors

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In brief

Yang et al. systematically interrogate selective advantages that carrying 18 tRNAs may convey to a T4-like Vibriophage and find that the main driver behind phage tRNA acquisition is the pressure to sustain translation as host machinery degrades, a process resulting in a dynamically adapted codon usage strategy during the infection course.

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Degradation of host translational machinery drives tRNA acquisition in viruses

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SUMMARY

Viruses are traditionally thought to be under selective pressure to maintain compact genomes and thus depend on host cell translational machinery for reproduction. However, some viruses encode abundant tRNA and other translation-related genes, potentially optimizing for codon usage differences between phage and host. Here, we systematically interrogate selective advantages that carrying 18 tRNAs may convey to a T4-like Vibriophage. Host DNA and RNA degrade upon infection, including host tRNAs, which are replaced by those of the phage. These tRNAs are expressed at levels slightly better adapted to phage codon usage, especially that of late genes. The phage is unlikely to randomly acquire as diverse an array of tRNAs as observed ($p = 0.0017$). Together, our results support that the main driver behind phage tRNA acquisition is pressure to sustain translation as host machinery degrades, a process resulting in a dynamically adapted codon usage strategy during the course of infection.

INTRODUCTION

The question of why some bacteriophages encode their own transfer RNAs (tRNAs) has been of interest since the late 1960s, when tRNAs were discovered to be carried by T4 (Daniel et al., 1968; Weiss et al., 1968). This finding countered the notion that bacteriophages should be under selective pressure to maintain compact genomes. Most phages simply make use of the host translational machinery, and thus, tRNA genes and other translation-related genes are often considered to be a hallmark of cellular life (Abergel et al., 2015; La Scola et al., 2003; Raoult et al., 2004; Schulz et al., 2017). Then, why do some phages carry tRNAs? This question is now more puzzling, as the recent explosion in phage genome sequencing has revealed many more cases of "jumbo phage" that carry tRNA and other translation-associated genes (Hatfull, 2008; Skliros et al., 2016).

There are currently several hypotheses as to why phages carry tRNA. For the T4 phage, almost all of its eight tRNAs correspond to codons that it uses more frequently than its host (Scherberg and Weiss, 1972), leading to the proposal that bacteriophages typically carry tRNA in order to bias translation toward their own genes. Additionally, Cowe et al. found evidence suggesting that the codon usage bias introduced by T4 tRNAs is especially pronounced toward its late genes (Cowe and Sharp, 1991). Experimentally, tRNA mutants of T4 are still able to replicate and lyse their hosts but show a moderate decrease in the burst size under some experimental conditions (Wilson, 1973). However, for another broad host-range T4-like phage KVP40, which also carries 25 tRNAs (Matsuzaki et al., 1992; Miller et al., 2003), the signal for codon usage bias optimization by phage tRNAs is less clear (Miller et al., 2003). In fact, this signal may be an artifact because bacterial tRNA levels are often highly optimized for their codon utilization (Ikemura, 1981; Sharp et al., 2010), and codon usage tends to be very species specific (Botzman and Margalit, 2011; Grantham et al., 1980). Hence, even phages that do not carry tRNAs commonly have noticeably different codon usage distributions than that of their hosts. It, therefore, remains an open question whether codon bias optimization is a strong enough driving force to explain phage carriage of tRNA genes. Moreover, other correlations have been described, such as larger phages carrying more tRNAs, and lytic phages being more likely to carry tRNA than temperate phages

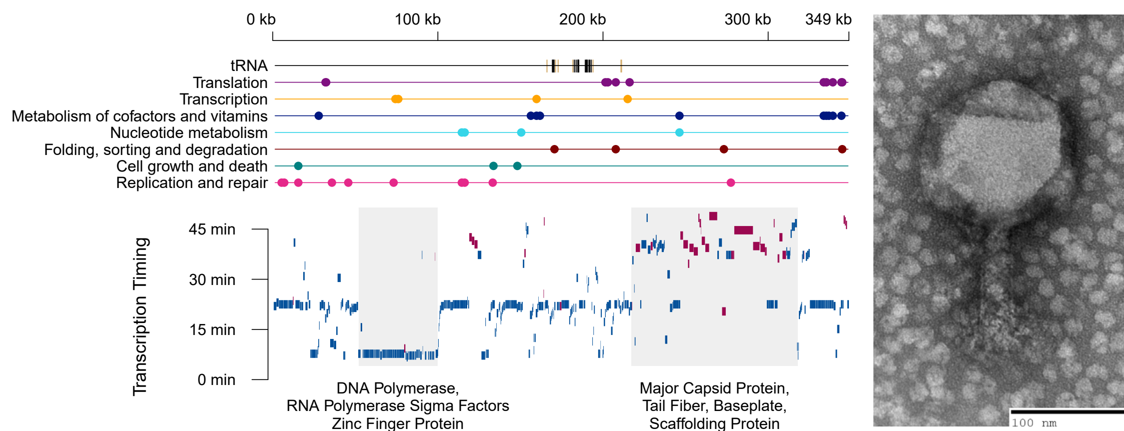

Figure 1. Phage 2.275.O carries 18 tRNA genes and is a large phage in both capsid size (120 nm) and genome size (348,911 bp)

(A) Estimation of transcriptional units and their timing of expression. The top plot shows the genome position of KEGG annotated genes (full list of annotations included in Table S1), and the bottom plot shows time to reach half the maximum expression of that gene (bottom). Blue and red bars indicate genes on the positive and negative strand, respectively. Early genes tend to be polymerases and sigma factors, while late genes tend to be structural proteins (full list of blast annotations included in Table S2). More detailed annotations can be found in Figure S1.

(B) Electron microscopy image of phage 2.275.O.

(Bailly-Bechet et al., 2007). Furthermore, through a process of elimination, Delesalle et al. (Delesalle et al., 2016) hypothesized that tRNAs help to sustain growth during infection or to expand the host range of the phage.

To address these hypotheses, we systematically explored the selective advantages that carrying tRNA might confer by performing an in-depth genomics and transcriptomics analysis on the infection characteristics of a broad host range (T4-like Vibriophage 2.275.O, carrying 18 tRNA genes) in its host of isolation (*Vibrio cyclitrophicus*, strain 10N.286.54.E11). We found that the phage tRNA participates in translation, but while there may be some codon optimization, this may not be the most important factor at play. Instead, the infection phenotype is all destructive in that within approximately the first 15 min of infection, the host genome is degraded, as is the host transcriptome. Therefore, there is little host RNA left against which to optimize codon usage bias. Rather, because the host tRNA is degraded as well, the phage must supply its own translational machinery in order to sustain its reproduction cycle. Finally, we show that the main factor optimized by the phage tRNA is the diversity of the tRNA array, which allows the large phage to sustain a longer replication cycle amid the decaying pool of host resources that result from the lytic infection cycle. This illustrates the potential for a positive feedback loop: large phages must degrade host machinery for parts, and hence, bringing their own machinery allows them to gain a competitive edge, which selects for even larger phages.

RESULTS

Genomic analysis of phage and host reveals differences in genomic codon usage patterns

In order to dissect the selective advantages that tRNA carriage may convey, we used phage 2.275.O [NCBI:txid1881285] as a model. This phage is a part of the Nahant Collection, an extensive collection of Vibriophages previously described by Kauffman et al. (Kauffman, 2014; Kauffman et al., 2018). At 348,911 bp, it is among the largest known bacteriophage (Figures 1 and S1), and it is capable of infecting hosts of at least two different species, *Vibrio cyclitrophicus* and *V. lentus*. Finally, its genome encodes for 18 tRNAs that correspond to 15 amino acids (excluding alanine, tryptophan, aspartic acid, histidine, and lysine), as well as another seven tRNA-like sequences with putative introns.

To test whether the codon usage hypothesis is plausible, we first conducted preliminary analyses to verify whether a codon usage difference between the phage and host exists. To this end, we applied multidimensional scaling (Figure 2A) and multinomial discriminant analysis (Figure 2B) to the codon usage for each gene from the genomes of the phage and its host of isolation. We observed that there was indeed a codon usage difference between the two organisms, allowing us to next ask whether the phage tRNAs bias translation in the direction of this difference. Previously, the tRNA copy numbers (Figure 3A) in the genomes of phages and their hosts were used to assess whether phage tRNAs may optimize codon usage differences (Bailly-Bechet et al., 2007). By comparing the odds ratio of each codon in the phage versus host genome (Figure 3B), it is clear that the codons that can be recognized by an anticodon from a host tRNA (according to extended wobble rules summarized by dos Reis et al. (Reis et al., 2004; Watanabe and Osawa, 1995; Yokoyama and Nishimura, 1995)) appear to be more commonly used by host genes than by phage genes. On the other hand, codons that can be recognized by both phage and host tRNAs span across the range of usage preferences. Instead of selectively acquiring tRNAs that are more beneficial to it than its host, it appears that the phage seeks to acquire diverse tRNAs but places a lower priority on those that benefit mainly its host.

However, this analysis does not account for RNA modifications, which can often be found at the wobble base and may thus change tRNA specificity; furthermore, the tRNA expression level may be more relevant information for assessing any translational bias that may be introduced.

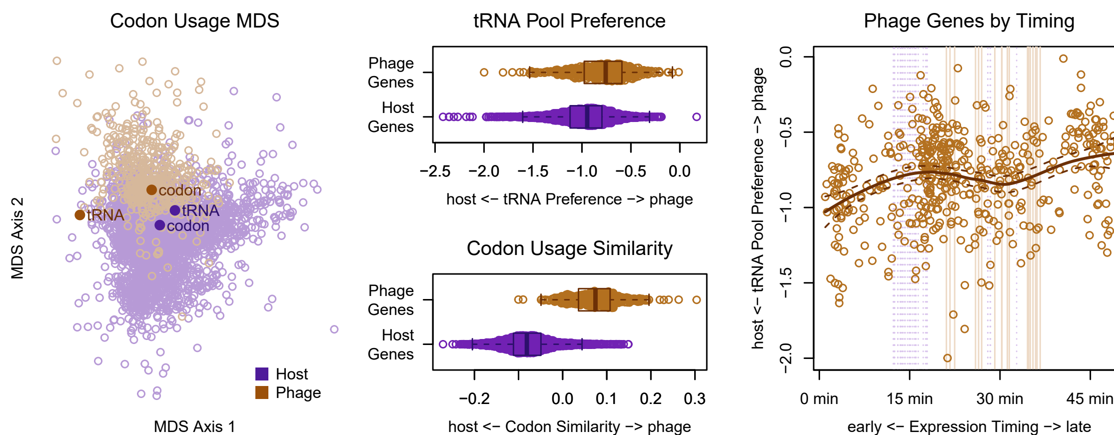

Figure 2. Codon usage bias introduced by the phage tRNA pool is more pronounced in late genes than in early genes

(A) A multidimensional scaling (MDS) plot of phage and host proteins using Shannon-Jensen Divergence of the codon distributions shows that codon usage difference between phage and host. Points representing the codon recognition capacities of the tRNA pool for each organism are overlaid. Points representing the average codon usage for each organism are also overlaid.

(B) Preference for the phage tRNA versus the host tRNA pool (slant). (Zero signifies ambivalence.).

(C) Mean codon usage for host and phage.

(D) Slant toward the phage tRNA pool versus timing of expression depicted as the center of mass of RNA expression for the first round of infection. Note that this is different from the expression timing described in Figure 1. The center of mass in this plot indicates how quickly the RNA transcript is degraded, while the time to half maximum expression shown in Figure 1 summarizes transcription timing.

tRNA sequencing suggests that phage tRNA actively participate in translation

Phage tRNAs are both expressed and modified, suggesting that they are involved in translation. We performed tRNA sequencing on an infection time course, sampled at 15-min intervals, which enabled us to infer post-transcriptional modifications on the phage tRNA transcripts, as required for translation. These modifications included the CCA tail, which allows for amino acid attachment as well as for successful interaction with the ribosome, and synthesis of the CCA tail is thought to be a step in tRNA quality control (Dupasquier et al., 2008; Hou, 2010; Korostelev et al., 2006). We observed that the tails of the five phage tRNAs whose genomic sequences do not end in CCA (Cys-GCA ends in CTA, Gly-TCC ends in CTA, Ile-GAT ends in CAA, Leu-TAA ends in CCG, and Tyr-GTA ends in CAA), were modified into CCA upon transcription (see also Figure S2 and STAR Methods). On the other hand, the genomic sequences of all host tRNA ended up with a CCA tail. Additionally, while the host carries a CCA modification protein in its genome (Genbank locus tag: NVP2275O_348), the phage carries its own CCA modification protein as well. Hence, the phage tRNAs appear to be processed such that they can participate in translation.

We were also able to infer the putative addition of similar base modifications on the phage and host tRNAs. In our tRNA sequencing protocol, we used the group II intron reverse transcriptase TGIRT (Clark et al., 2016), which can read through RNA modifications but may leave DNA base substitution signatures evident as a multinomial mixture of A, C, G, T, and/or a decrease in reading density downstream of the modification (see also Figure S3 and STAR Methods). For example, by comparing one of the phage CAU tRNAs (Genbank genome location: 182648–18272) with a host CAU tRNA (Genbank locus tag: BCV12_11325), both were modified with 4-thiouridine on the 8th base, 3-(3-amino-3-carboxypropyl)uridine on the 47th base, and, importantly, 2-lysidine on the 34th base (see also Figure S3 and STAR Methods) putatively changing them to AUA-recognizing isoleucine tRNA (Harada and Nishimura, 1974). Other similar putative modifications could be observed between phage tRNA and their host analogs, for example, 5-carboxymethylaminomethyl-2-thiouridine on base 34 of Glutamine tRNA, 1-methylguanosine on base 37 of (most) Leucine tRNA (supplemental information). These similarities suggest that phage tRNAs may be recognized and processed by the same enzymes as corresponding host tRNAs.

Interestingly, we also observed seven intron-containing tRNA-like sequences. These, while not recognized by tRNAscan-SE (Lowe and Eddy, 1997), were recognized by another tRNA caller, Aragorn (Laslett and Canback, 2004). These sequences are only expressed at levels as low as 0.003 times (as in the case of threonine tRNA) to 0.2 times (as in the case of serine tRNA) the abundance of an isoacceptor phage tRNA without an intron. Although a small fraction of the reads did appear to be spliced as called, many did not, and the aligned anticodon loop was fairly heterogeneous for these species. In addition, many of these sequences do not end in CCA and did not appear to receive CCA tails. These intron-containing tRNA-like sequences may serve non-canonical functions, and were therefore not used beyond the initial preliminary analyses.

Codon usage bias is present but not pronounced

Having found evidence supporting the idea that the 18 phage tRNAs without introns likely participate in translation, we tested the most common hypothesis as to why phages carry tRNA—to increase the translational efficiency of their own genes over that of their hosts' (Bailly-Bechet et al., 2007; Enav et al., 2012; Scherberg and Weiss, 1972; Wilson, 1973). For each gene, we calculated a value representing the efficiency with which it can be translated by the phage tRNA pool, relative to the efficiency with which it can be translated by the host tRNA pool (see

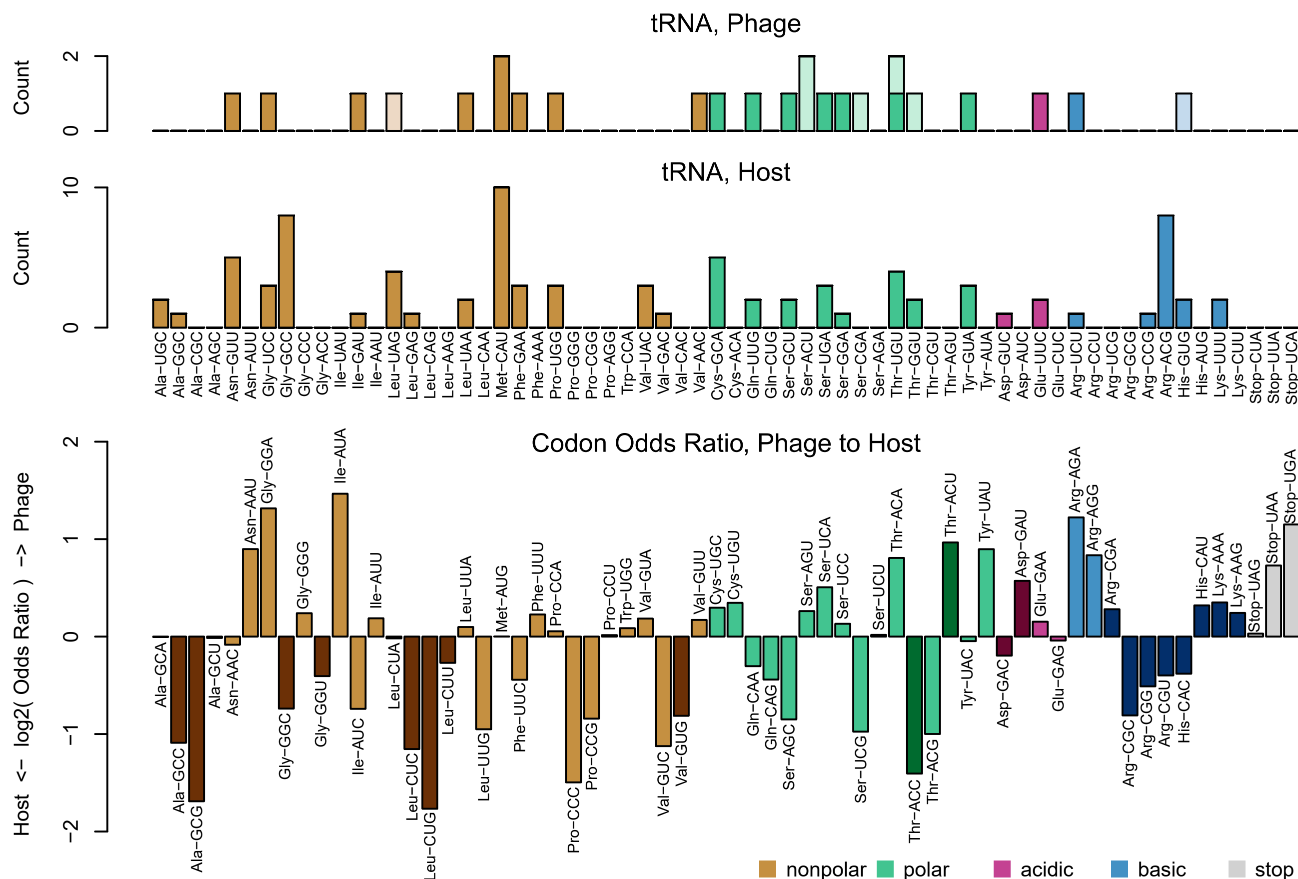

Figure 3. Analysis of the phage and host genome supports the codon usage hypothesis

(A) tRNA content in the genomes of the phage and host. Less saturated bars indicate putative tRNA with introns.

(B) Differences between the codon usages of phage and host. Darker-hued bars indicate codons that cannot be recognized by phage tRNA, given the wobble rules summarized by dos Reis et al.

STAR Methods). For simplicity, we will refer to this value as the "slant" of a gene.

We observed that the slant of the phage genes was slightly more in the direction of the phage tRNA pool than was the slant of the host genes (Figure 2C). However, this effect was weak compared with a more optimal axis of discrimination, which is defined by the average gene codon usage for each organism (Figure 2B). The statistical significance of this small effect (KS test: $p < 2.2e\text{-}16$) seems unreasonable; in fact, we must re-evaluate this test in the context of the problem at hand. Specifically, we already know that a codon usage bias exists between the host and phage genes, and we also know that host tRNAs are closely matched to the host codon usage (Figure 2A). Therefore, almost any randomly chosen set of tRNA will betray a codon usage difference between the phage and host genes. Then, it is simply a coin flip as to whether the difference is in the direction of the host tRNA pool or the phage tRNA pool.

We therefore asked whether the slant values for phage versus host genes are different, given the known difference in codon usage for the two organisms. Conditioning appropriately, the probability of seeing a high or a higher difference in slant between the phage proteins and host proteins in the direction of the phage tRNA pool was approximately 0.08. (see STAR Methods and Figure S4) This probability is, at best, suggestive, but we cannot be confident that codon usage bias optimization has been the main factor driving tRNA acquisition.

We next calculated the codon usage profiles for other *Vibrios* in the Nahant Collection for which we have completely sequenced genomes, including *Vibrio lentus* and additional *Vibrio cyclitrophicus* strains that are known hosts of 2.275.O. As expected, we found that codon usage profiles clustered by species (see STAR Methods and Figure S5, top panel). *V. cyclitrophicus* hosts are more similar in codon usage to 2.275.O than *V. lentus* hosts. (see STAR Methods and Figure S5, bottom panel) As *V. cyclitrophicus* was the host of isolation, one could posit that *V. cyclitrophicus* might be a "preferred" host for 2.275.O. However, we note that we cannot exhaustively identify all potential hosts for this phage.

Host genomic DNA and RNA degradation as the driving factor for tRNA acquisition

One potential explanation for the low overall signal for codon optimization is that only a subset of the phage genes is affected, in particular, the late genes, as previously shown for T4 (Cowe and Sharp, 1991). Codon usage optimization toward

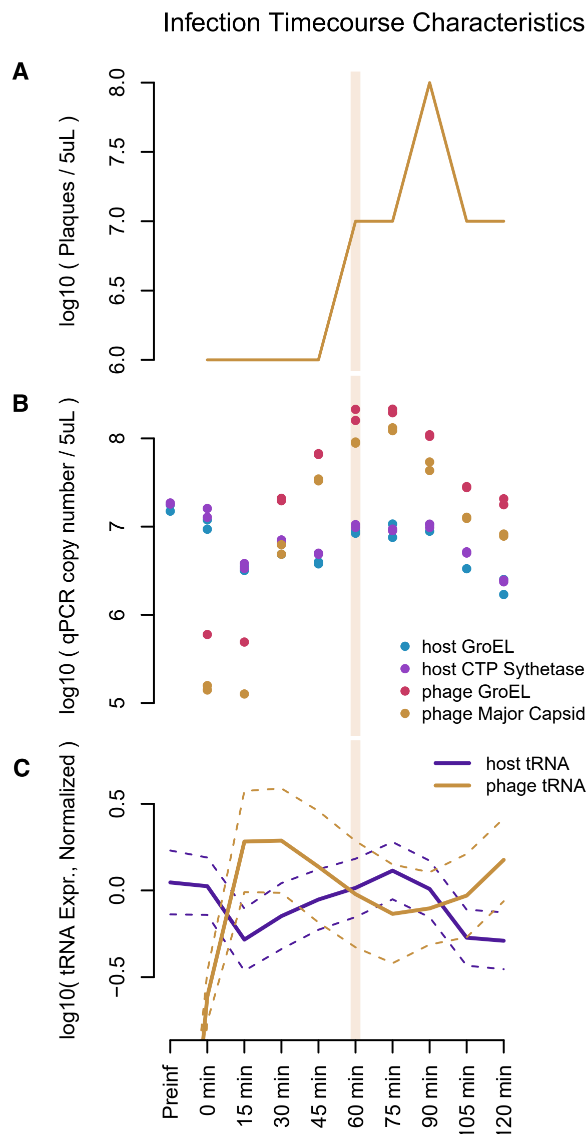

Figure 4. Time course of phage 2.275.O. infection

Approximately a tenth of the hosts remain uninfected and begin to grow again. A second round of infection starts at approximately 90 min.

(A) Phage plaques begin to appear 30 min into infection, and peak at approximately 90 min.

(B) qPCR results to quantify bacterial chromosome copies show that the host genome is degraded rapidly upon infection.

(C) The tRNA subset of RNA-seq shows that host tRNAs, shown in purple, are degraded upon infection while phage tRNAs, shown in orange, increase. An average has been taken over all tRNA species, and the errors shown are 1.96 × standard dev of the log mean. Reads are normalized to a firefly luciferase spike-in for each sample.

the late genes might be advantageous for a few reasons: (1) mRNAs from the earliest genes might already be undergoing translation and degradation as the phage tRNAs are transcribed, and therefore must utilize mainly the host tRNA pool; and (2) the host tRNA pool might degrade, in which case translation during the late stages of infection might heavily rely on phage tRNA.

Some evidence in the literature supports the latter hypothesis. During T4 infection of *E. coli*, degradation of host DNA is initiated by Endo II and Endo IV (Miller et al., 2003), in part, to help supply the nucleotide pool for phage replication. This comes with a consequence: although tRNAs tend to be more stable than other RNAs (Davis et al., 1986), they can undergo rapid degradation under stress conditions (Svenningsen et al., 2017; Zhong et al., 2015). In fact, during T4 infection, *E. coli* uses nucleases to deplete its own lysine tRNA, dialing down translation, seemingly in defense, while, as a ‘‘rebuttal,’’ T4 RNA ligase is able to repair damaged tRNA (Amitsur et al., 1987). The evidence for this all-destructive infection phenotype suggests that supplying translational components might help the phage to fill the growing gaps in host machinery and thereby prolong the replication period.

To test whether phage 2.275.O infection is similarly all destructive, we used qPCR to check whether host DNA was degraded upon infection. We found that the genomic copy number of the host genes probed for (GroEL and CTP Synthetase) dropped by approximately 80% within the first 15 min of infection, which is in line with expectation given the number of infected cells in our assay (Figures 4A and 4B, additional validation is also presented in Figure S6). On the other hand, the phage production does not occur until approximately 30 min into the infection. Because the host genome is degraded, tRNA can no longer be produced from the host genome, and if the host tRNA is degraded as well, tRNA might become a limiting resource for translation during the late stages of infection.

When examining the tRNA expression from transcriptome sequencing data, we found that host tRNAs were indeed degraded rapidly, reaching a minimum value at around 15 min, whereas phage tRNAs were continually produced (Figures 4C and S7, related to STAR Methods for individual tracts of host and phage tRNAs by amino acid). The increase in host RNA after 15 min is likely due to the regrowth of uninfected cells in the culture. We note that the presence of uninfected cells in the culture renders it difficult to reliably infer the levels of host tRNA in the infected cells.

These observations support the hypothesis that, as the host tRNA pool is degraded, the phage tRNA allows translation to be sustained; this may especially benefit the late genes, which do not reach half their maximum expression until 40–45 min into the infection (Figure 1).

Having found that the host genome and transcriptome (including the tRNA) were indeed degraded during infection, we next utilized the full transcriptome sequencing data to quantify 2.275.O gene expression timing in order to assess whether late genes, which have a greater necessity for relying on phage translational machinery, were more adapted to the phage tRNA pool than early genes. In fact, we did observe that the slant of the late phage genes was further in the direction of the phage tRNA pool than the slant of the early genes (Figure 2D). However, the absolute slant of even the late genes was closer toward the host tRNA pool than the phage tRNA pool, implying that while suggestive, codon usage bias optimization might not be the driving force for phage acquisition of tRNAs.

Prolonging the replication period amid host cell shutdown

If the phage tRNAs are optimized for the ability to sustain translation in the absence of the host tRNAs, we would expect the

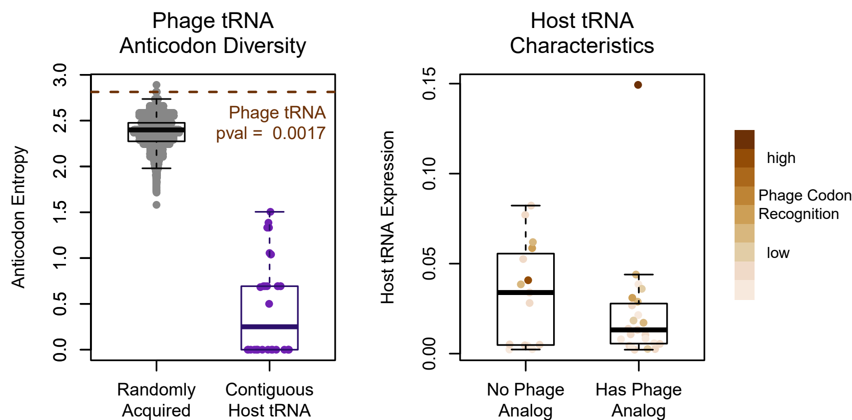

Figure 5. tRNA carried by the phage may supplement the degrading pool of host tRNA

(A) The probability of selecting, uniformly at random from the host genome, a tRNA array that is able to encode as many anticodons as that carried by the phage is 0.0017. In addition, contiguous stretches of tRNA in the host genome, which are typically thought to be the result of duplication events, encode very lowly diverse anticodons. The phage's tRNA collection, therefore, appears to be the result of multiple acquisition and selection events.

(B) Of the tRNA not carried by the phage, most are highly expressed by the host, and others correspond to codons not very highly used by the phage genome.

phage to carry as diverse an array of tRNAs as possible. In fact, it is striking that the 18 phage tRNAs without introns each represent different anticodons (there are two CAU anticodons, however, one of these is likely modified by 2-lysidine, making it an AUA-recognizing leucine as opposed to an AUG-recognizing methionine). In simulating draws of tRNA from the host genome, we found that the tRNA carried by 2.275.O is more diverse in anticodons encoded than would be expected at random (Figure 5A, p = 0.0017).

This observation was even more striking when considering that neighboring tRNAs within the host genome generally have very low diversity, as they are likely the result of gene duplication events and tend to code for the same amino acid (Figure 5A). This indicates that picking up as diverse an array of tRNAs as observed in the 2.275.O genome was not a matter of a few simple recombination events, but many. Thus, the diversity of this tRNA array appears to be under high selective pressure.

And what of the tRNAs that the phage does not carry? We found that the host expresses many of these tRNAs in high abundance (Figure 5B). Assuming similar rates of degradation for each tRNA, the more highly expressed tRNAs may persist longer during infection, thereby reducing the selective pressure for the phage to acquire its own copies. The tRNAs without phage analogs that are expressed lowly by the host recognize codons that are used very infrequently by the phage. These two types of tRNA may confer less of a selective advantage to the phage than the tRNA already present in the phage, implying that for these tRNAs the phage has reduced its dependence on their codons rather than acquiring its own copies of the tRNAs. Taken together, the observations presented in this study strongly imply that the primary function for phage tRNAs is to supplement degrading host translational machinery, which results from an all-destructive lytic infection phenotype.

DISCUSSION

Our results indicate that the main role of the phage 2.275.O tRNAs is to support the translation of this large lytic phage as the host cell shuts down. Upon 2.275.O infection, host genomic DNA is degraded, as are mRNA transcripts. Degradation reaches a baseline around 15 min; however, phage particles are only released starting around 30 min after the onset of the infection, implying that without phage tRNA production, late genes might experience resource limitation during translation. Although the tRNA array of phage 2.275.O does not appear to optimize tRNA/codon usage bias toward its genes, on the whole, we do observe that the codon usage of the phage genes expressed late during the infection is more in the direction of the phage tRNA pool than that of the early genes. Additionally, the diversity of the phage tRNA array appears to be optimized, implying that the main selective force at play is a drive toward self-sufficiency in the wake of host degradation.

This simple line of logic unifies many observations previously made in the literature, either through deep interrogations of the T4 infection cycle or broad analyses of tRNA-carrying phages. First, the presence of many tRNAs is more often found in the genomes of lytic phages than those of temperate phages (Bailly-Bechet et al., 2007). Aggressively lytic phages often degrade the host genome as the phage can then use the nucleotides to increase its burst size. But because translation is required for phage particle production, these phages presumably benefit from shuttling their own translation machinery by extending the replication period beyond the time at which the host resources are depleted.

A second observation is that phage tRNAs appear to optimize codon usage bias toward phage genes and away from host genes (Scherberg and Weiss, 1972). According to our findings, tRNAs absent from the phage correspond to those that tend to be more highly expressed in the host than tRNAs present in the phage, and tRNAs highly expressed in the host correspond to codons that are most commonly used by the host or are most biased toward the host (Figure S7, related to STAR Methods). This backdoor correlation may explain a large part of the observed codon usage bias effect. Moreover, once the phage acquires tRNA, an adaptive feedback loop can form between the phage's genome codon usage and the tRNA array that it carries. This feedback loop explains a third observation, which is that codon usage bias is more pronounced in late phage genes than in the early genes (Cowe and Sharp, 1991; Kunisawa, 1992; Kunisawa et al., 1998). Early mRNAs require the use of host tRNAs, as the bulk of phage tRNAs are still in the process of being transcribed and processed, whereas late mRNAs are expected to be more dependent on the phage tRNAs, as the bulk of the host tRNA might be degraded later in the infection.

Finally, a fourth observation is that phages with tRNAs generally have larger genomes than those without (Bailly-Bechet et al., 2007). For phages with large genomes, it is more important to degrade the host genome to free up nucleotides, leading again to the quandary presented by the first point. In addition, a larger phage might require longer periods of replication and more resources for supporting translation in the wake of degrading host tRNA. The longer the infection persists past host resource degradation, the stronger the selective pressure for the phage to encode their own machinery, which in turn selects for larger phage. A tRNA deletion mutant of phage 2.275.O could address the key questions of how tRNAs change the latent period and burst size during infection. We note that this experiment may prove very difficult to do in a wild, non-model system (such as the one that we used here) but could potentially be carried out in a more tractable model system.

Using this lens, it is interesting to compare 2.275.O against its foil in the same phage sampling collection, the Autolykiviridae (Kauffman et al., 2018). In contrast with this 349-kb phage, Autolykiviridae carry a particularly small, streamlined genome, at only 10 kb. With such small genomes, there may be less selective pressure to free up the nucleotide pool. In fact, Autolykiviridae do not degrade the host genome. The infection cycle of these viruses can last in the order of weeks, and with only 20 genes in its genome and no known translation supporting functions, they must rely entirely on the host translation machinery. Although a whole spectrum of strategies might co-exist, we can see two extreme and contrasting strategies here.

This type of phage infection might have convenient applications for studying tRNA regulation. Although translation is fundamental to all of life, many aspects are still unknown. For example, while some tRNA modifications have been shown to be necessary for correct folding, synthetase recognition, degradation, and translation regulation (Lorenz et al., 2017), the functions of most modifications are unknown (Kirchner and Ignatova, 2015). Many recent findings about tRNA are conducted in systems in which a cellular stress response involving tRNA can be triggered (Kirchner and Ignatova, 2015). Lytic phage infection offers a similar convenience in that it can be synchronized through a one-step-growth experiment (Ellis and Delbrück, 1939). Because of this, tRNA can be "tracked" from the newly synthesized nascent form to processed intermediates and degraded products. In fact, this can be seen in our tRNA sequencing time course (Figures S8–S11, related to STAR Methods). Exciting new technologies for probing translation now exist, such as ribosomal footprint profiling (Ingolia et al., 2009), tRNA-ribo-seq (Chen and Tanaka, 2018). Work in combining these techniques with phage growth experiments may be a promising future direction for uncovering further insights into tRNA processing and use in translation.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cels.2021.05.019.

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AUTHOR CONTRIBUTIONS

D.P.B., M.P., L.K., J.Y.Y., and W.F. designed the project. J.Y.Y., W.F., K.M.K., F.M.-S., J.M.B., and C.M.A. conducted all experiments. M.P., L.K., and J.Y.Y. wrote the paper. All authors approved the final paper. J.Y.Y. and W.F. contributed equally to the paper and are co-first authors. M.P. and L.K. contributed equally to the paper and are co-corresponding authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
T4-like Vibrio phage 2.275.O._10N.286.54.E11	Martin Polz Lab	MG592671.1
Vibrio cyclitrophicus, strain 10N.286.54.E11	Martin Polz Lab	NZ_MCTE00000000.1
Critical commercial assays		
Ribo-Zero Bacteria kit (gram negative and positive)	Illumina	discontinued
Kapa Hyperprep	Roche	KK8504
Pippin Prep	Sage Science	No catalog #
RNA Clean & Concentrator	Zymo Research	R1080
TGIRTTM-III enzyme	InGex	TGIRT50
RNA Ligase 2, truncated KQ	New England Biolabs	M0373S
5 DNA Adenylation Kit	New England Biolabs	E2610L
Oligo Clean & Concentrator	Zymo Research	D4060
KAPA SYBR FAST	Roche	KK4600
MinElute PCR Purification Kit	Qiagen	28004
Deposited data		
tRNA-seq	Raw sequencing reads	PRJNA524872
RNA-seq	Raw sequencing reads	PRJNA524877
Oligonucleotides		
3′ preadenylated adapter AppNNN NAGATCGGAAGAGCACACGTCT/ iBiodT/iBiodT/3ddC/	This paper	N/A
qPCR primers, GroEL, host genome: CAATGGATCTTAAGCGCGGC and CAGAGATAACCGTACCGCCC	This paper	Genbank locus tag BCV12_01410
qPCR primers, CTP synthetase, host genome: CTTTGGCGATCGTGGTGTTG and TTTTCTAATTCGCCGCGCTG	This paper	Genbank locus tag BCV12_03025
qPCR primers, GroEL, phage genome: CTTTGAAGACATGGGCGCAC and AACGACTAGGGTTGCAAGCA	This paper	Genbank locus tag NVP2275O_355
qPCR primers, Major Capsid Protein, phage genome: TGAAGGTGTTATGGGTCG CC and ATACGGGCAGTAGAACGCAG	This paper	NVP2275O_445
preadenylated DNA adapter AppNNNNGAT CGTCGGACTGTAGAACTCTGA/3ddC/	This paper	N/A
Sequencing primer AATGATACGG CGACCACCGAGATCTACACGTTCAGA GTTCTACAGTCCGACGATC	This paper	N/A
Sequencing primer CAAGCAGAAGACGG CATACGAGATBBBBBBGTGACTGGAGT TCAGACGTGTGCTCTTCCGATCT (BBBB BB indicates barcode sequence)	This paper	N/A
Software and algorithms		
LocaRNA	Will et al. (2007)	http://www.bioinf.uni-freiburg.de/Software/LocARNA/
tRNAscan-SE	Lowe and Eddy (1997)	http://lowelab.ucsc.edu/tRNAscan-SE/
Aragorn	Laslett and Canback (2004)	http://lowelab.ucsc.edu/tRNAscan-SE/
MUSCLE	Edgar (2004)	https://www.drive5.com/muscle/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Libusha Kelly (libusha.kelly@einsteinmed.org).

Materials availability

Phage and host are available from Martin Polz (martin.f.polz@univie.ac.at).

Data and code availability

Source data statement

Raw sequencing reads are available on the NCBI Sequenced Reads Archive under BioProject numbers PRJNA524872 (tRNA-seq) and PRJNA524877 (full transcriptome RNA-seq).

Code statement

This paper does not report original code.

Scripts statement

Scripts to generate the figures reported in this paper are available in the R (R Core Team, 2018) package and their use is described in vignettes included with the package (https://github.com/ratatstats/tRNAbias). Please first install the package *devtools*, and then from R, run following command: devtools::install_github("ratatstats/tRNAbias", build_vignettes = TRUE, force = TRUE)

Any additional information required to reproduce this work is available from the lead contact.

METHOD DETAILS

Exploratory genome-based codon usage bias analysis

In order to assess the plausibility of the codon usage bias hypothesis, we conducted a preliminary analysis of the phage and host genomes. The tRNA carried by each organism was called using tRNAscan-SE (Lowe and Eddy, 1997) and Aragorn (Laslett and Canback, 2004). The multidimensional scaling analysis uses Shannon-Jensen divergence between codon distributions for each protein as the distance metric. The odds ratio for each codon is defined as $\frac{P(codon \mid org = phage)/(1-P(codon \mid org = phage))}{P(codon \mid org = host)/(1-P(codon \mid org = host))}$.

tRNA sequencing timecourse

In order to explore the shift in tRNA abundance throughout the course of infection, we conducted a one-step-growth experiment and collected samples at 15 minute intervals. Cells from the same culture were split in two (control vs. infection) then centrifuged to pellets. The control sample was resuspended in 200 μL of Difco 2216 Marine Broth, and the infection sample was resuspended in 150μL 2216 and 50 μL of phage lysate. The samples were left to sit for 5 minutes to allow for adsorption of the phage, then diluted to a volume of 15 mL in order to deter further infection. This total volume was split in 5, one sample from each set was immediately centrifuged and flash frozen as a "time 0" sample, and the rest were placed on a shaker, then centrifuged down and flash frozen at 15 minute intervals. 500 μL aliquots were taken from each sample prior to centrifugation to be plated as a spot check of phage concentration. A schematic of this experiment is presented in Figure S12, and the resulting plaque data is presented in Figure S13.

RNA sequencing timecourse

To further hone in on particular genes that the codon usage bias may favor, a second phage growth experiment was collected for full-transcriptome RNA sequencing. In this experiment, an aliquot of a culture was centrifuged and flash frozen as a preinfection timepoint, the remainder of the culture was then centrifuged to a pellet then resuspended with 400 μL of a phage lysate. The samples were left to sit for 5 minutes to allow for adsorption of the phage, then diluted to a total volume of 45 mL in order to deter further infection. This total volume was split into 9 samples of 5 mL each, to be taken in 15 minute intervals from time 0 to 120 minutes. The sampling procedure involved flash freezing 3 pellets spun down from 1.5 mL aliquots of each sample, then immediately doing serial dilutions of unfiltered and filtered viruses to assess phage growth and stage of infection. A schematic of this experiment is presented in Figure S14, and the resulting plaque data is presented in Figure S15

Total RNA extraction

Total RNA from the infection time series (flash-frozen pellets) was extracted by the hot phenol method. Briefly, cell pellets were resuspended in TE (10 mM Tris pH 7.0, 1 mM EDTA) and treated with 0.5 mg/ml lysozyme at room temperature for 5 minutes. Then NaOAc (100 mM final concentration) and SDS (1% final concentration) were added, followed by an equal volume of acid phenol: chloroform pH 4.5 (ThermoFisher). The mixture was shaken at 65°C for 10 minutes using a thermomixer, and centrifuged at 20000 x g for 5 minutes. The upper phase was washed by chloroform and centrifuged at 20000 x g for 5 minutes. The phenol/chloroform extraction was repeated once, and the upper phase was precipitated with isopropanol and 300 mM NaOAc. Precipitated RNA was washed with 75% ethanol, air dried and resuspended in water.

High-throughput sequencing of tRNAs

tRNAs were gel purified from total RNA on a 10% urea polyacrylamide gel (size selected between 70 and 100 nt). Gel pieces were macerated and soaked in 0.3 M NaCl overnight with rotation at 4°C for elution. tRNAs were precipitated with isopropanol using linear acrylamide as the carrier. RNA pellets were resuspended in 100 mM Tris-Cl pH 9.5 and incubated at 37°C for 1.5 hours for deacylation. After deacylation RNA was purified using Oligo Clean & Concentrator (Zymo Research) and eluted in 10 mM Tris pH 8.0. Purified RNA was ligated to a 3' preadenylated adapter AppNNNNAGATCGGAAGAGCACACGTCT/iBiodT/iBiodT/3ddC/ (final concentration 10 uM) using RNL2 truncated KQ (NEB) with 10% PEG8000 at room temperature overnight. After ligation RNA was purified using the MinElute PCR Purification kit (Qiagen) and reverse transcribed using TGIRT^{TM}-III enzyme (InGex) under manufacturer's instructions. Briefly, RNA was incubated with an RT primer AGACGTGTGCTCTTCCGATCT (0.1 μM final concentration) and RT buffer at 85°C for 5 minutes, and cooled to 25°C at 0.1°C per second. DTT and TGIRT^{TM}-III were added and the mixture was incubated at room temperature for 30 minutes. dNTPs were added and the reaction was incubated at 60°C for 30 minutes. RNA was hydrolyzed by NaOH, neutralized by HCl and purified using MinElute PCR purification kit. cDNA was ligated to a preadenylated DNA adapter AppNNNNGATCGTCGGACTGTAGAACTCTGA/3ddC/ (preadenylated by 5' DNA adenylation kit (NEB)) using thermostable 5' App DNA/RNA ligase (NEB) following manufacturer's protocol (ligated at 65°C for 5 hours and heated inactivated at 90°C for 3 minutes). cDNA was purified using MinElute PCR purification kit, and amplified using KAPA HiFi HotStart PCR kit (Roche). PCR primers were AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC and CAAGCAGAAGACGGCATACGAGATBBBBBBGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT (BBBBBB stands for barcode sequence). PCR products were size selected between 150 nt and 260 nt using Pippin Prep (Sage Science), and sequenced on an Illumina HiSeq under 100 bp by 100 bp paired-end mode.

Full transcriptiome RNA-seq

For quantitative RNA-seq, 1.5 ng Firefly luciferase mRNA and 0.015 ng of Renilla luciferase mRNA was added to each cell pellet before hot phenol extraction. As each sample was derived from the same infection batch, which was distributed in equal volumes, these two luciferase mRNA spike-ins are proportional to the starting quantity of infected cells. Total RNA was treated by TURBO DNase (ThermoFisher) according to manufacturer's protocol and purified using RNA Clean & Concentrator kit (Zymo Research). mRNA was isolated using the Illumina Ribozero prokaryote kit (gram positive and negative, Illumina) and was prepared into libraries using the Kapa Hyperprep kit (Roche) following manufacturer's protocols. Libraries were sequenced on an Illumina HiSeq under 40 bp single-end mode.

Host gene expression data are displayed and clustered in Figures S16, S17, and Table S3.

Calling modifications on tRNA sequencing data

For the dual purposes of 1. assessing whether the phage tRNA are functional tRNA that participate in translation and 2. more accurately assigning wobble base affinities, RNA modifications were called based on reverse transcriptase substitutions. First, a reference alignment of phage and host tRNA was made. LocaRNA (Will et al., 2007), which accounts for RNA secondary structure, was used to make a first-pass multiple alignment. Phage tRNA called with introns often aligned poorly, and so the alignments were fixed using the putative secondary structures provided by Aragorn. The variable loops were aligned separately using MUSCLE (Edgar, 2004), then stitched back into the tRNA alignment. The final multiple alignment of the unique tRNA is shown in Figure S18. Next, *E. coli* tRNA from the MODOMICS database (Boccaletto et al., 2018; Dunin-Horkawicz et al., 2006) were aligned to this reference in order to identify what types of post-transcriptional modifications may be present. Reads from tRNA sequencing were then aligned to this reference using the affine gap penalty method "gotoh" provided by the *align.seqs*() function in mothur, version 1.34.4 (Schloss et al., 2009), with the following scoring: match=2, mismatch=0, gapopen=-5, gapextend=-1. Phage and host tRNA are sufficiently different such that reads can be mapped back to the originating organism (Figure S19). And finally, the base distribution at each position was used to fit a model for how modifications correspond to "missequenced" reads (as many of these are likely the result of base substitutions inserted by the reverse transcriptase upon encountering a modified base).

Calculating phage RNA expression timing

Instead of classifying phage genes into distinct categories of expression timing, continuous scales were defined. Because the host RNA expression level climbs until 75 minutes, this is taken to be the timepoint before the second round of infections begin. In order to get a sense of transcription and degradation for the purposes of the analyses presented in Figure 2, the phage RNA expression timing was then defined as the center of mass of the expression levels over time. Each RNA is color coded by its expression timing in Figure S20. For the purposes of visually identifying what may be transcriptional units within the genome (Figures 1 and S1), another measure of expression timing - the time taken to reach half the maximum level of expression - was defined.

Assessing Genome Degradation

Two genes were selected for qPCR to assess whether the host genome is degraded upon infection: GroEL (Genbank locus tag BCV12_01410, primers: CAATGGATCTTAAGCGCGGC and CAGAGATAACCGTACCGCCC) and CTP synthetase (Genbank locus tag BCV12_03025, primers: CTTTGGCGATCGTGGTGTTG and TTTTCTAATTCGCCGCGCTG). The phage genes, GroEL (Genbank locus tag NVP2275O_355, primers: CTTTGAAGACATGGGCGCAC and AACGACTAGGGTTGCAAGCA) and the Major Capsid Protein (Genbank locus tag NVP2275O_445, primers: TGAAGGTGTTATGGGTCGCC and ATACGGGCAGTAGAACGCAG), was also

assayed for contrast. Each sample was prepared using the Kapa SYBR Fast kit according to the manufacturer's instructions. A standard curve for each primer was made on 10 two-fold serial dilutions of each genome. This was then used to convert the CT values to copy number.

Codon usage bias analysis

In order to assess whether the phage tRNA pool may introduce translational bias toward its own genes, a summary statistic for each gene that represents the efficiency with which it can be translated by the phage tRNA pool, relative to the efficiency with which it can be translated by the host tRNA pool was calculated. This value, essentially a likelihood ratio, is referred to as the "slant" of a gene for brevity. The calculation is as follows:

To set up the analysis, the codon usage preference of a tRNA pool must first be calculated. In particular, it is defined as an estimated probability of a random codon being bound given the tRNA pool:

$$P(\text{codon}|\text{pool}=\text{host}) = \sum_x P(\text{codon},\ \text{tRNA}=x|\text{pool}\ =\ \text{host})$$
$$= \sum_x P(\text{codon}|\text{tRNA}=x,\ \text{pool}\ =\ \text{host})P(\text{tRNA}=x|\text{pool}=\text{host})$$
$$= \sum_x P(\text{codon}|\text{tRNA}=x)P(\text{tRNA}=x|\text{pool}=\text{host})$$

Here, $P(tRNA = x|pool = host)$ is defined as the read abundances from tRNA sequencing, normalized to each organism. $P(codon|tRNA = x)$ is defined according to revised wobble rules noted by Murphy, et al. (Murphy and Ramakrishnan, 2004; Watanabe and Osawa, 1995; Yokoyama and Nishimura, 1995), accounting for wobble base modifications inferred through tRNA sequencing results. Low affinity pairings receive a third the weight of high affinity parings.

These two points define a path that the codon distribution for each protein can, essentially, be projected upon in order to calculate a tRNA pool preference for each protein (here, referred to in short as the "slant"). Specifically, for each protein, the slant for a given gene with codon counts y, is calculated as the log likelihood ratio of observing the codons from that gene given the coding capacity of the phage tRNA pool vs. given the coding capacity of the host tRNA pool (divided by the total number of codons, to make the value more easily comparable among proteins):

$$\frac{1}{n}\Lambda(y) = \frac{1}{n}\log\left(\frac{\frac{n!}{y_1!\ldots y_k!}p_1^{y_1}\ldots p_k^{y_k}}{\frac{n!}{y_1!\ldots y_k!}h_1^{y_1}\ldots h_k^{y_k}}\right) = \frac{1}{n}\sum_{\text{codons } c} y_c \log\frac{p_c}{h_c}$$

where $p_c = P(codon\ =\ c|pool\ =\ host)$ and $h_c\ =\ P(codon\ =\ c|pool\ =\ phage)$. The slant is 0 if the two multinomial probabilities are equal, which can be interpreted intuitively as the gene having equal efficiencies of coding by the pool of phage tRNA and the pool of host tRNA. A nice property of this calculation is that if y is exactly np (if the codon usage of a gene matches exactly the coding efficiency of the phage tRNA pool), then the slant is $KL(p||h)$, or the Kullback-Leibler divergence between p and h. And if y is exactly nh (if the codon usage preference of a gene matches exactly the coding efficiency of the host tRNA pool), then the slant is $-\ KL(h||p)$, or the negative Kullback-Leibler divergence between h and p. However, it is possible for the slant to be less than $-KL(h||p)$ if the codon distribution for a given protein is even further away from the coding efficiency of the phage tRNA pool than that of the host tRNA pool. And likewise, the slant can be greater than $KL(p||h)$ if the codon distribution for a given protein is further away from the coding efficiency of the host tRNA pool than that of the phage tRNA pool.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of experiments and all software used can be found in the results section. Details of assessing the statistical significance of codon usage bias and tRNA diversity analysis are provided below.

Assessing the statistical significance of codon usage bias

A one-sided Kolmogorov-Smirnov test was used to assess the difference in distributions of slant values for phage genes vs. slant values for host genes. This resulted in a suspiciously low p-value (less than 1e-22), which was taken as a signal that the null model used may not have been fair in the context of this problem, and more relevant assumptions should be specified. In this case, we already know from preliminary analyses that the distributions of codon usage between phage and host genes is different (Figures 2C and 4B), so any randomly chosen vector of phage tRNA expression is likely to betray this difference.

Instead, it is more appropriate to ask whether the slant values for phage genes vs. the slant values for host genes are different *conditioning on the known codon usage distribution of the two organisms*. This was done using the following resampling scheme: First, 18 tRNA (the number of phage tRNA) are randomly sampled with replacement from the host genome, then a random expression vector for these 18 tRNA was generated by normalizing exponentially distributed random variables. This expression vector was used as a random phage tRNA expression vector. Then, based on this random phage tRNA expression vector and the known host tRNA expression vector, slant values were calculated for phage and host genes, and a one-sided KS-test was conducted on these slant

values. This procedure was replicated 200 times, and these test results formed the null distribution against which the original test result was compared.

tRNA array diversity analysis

The diversity of the tRNA pools was defined as the Shannon entropy of the amino acids encoded by the tRNA for each organism. The simulated randomly acquired diversity depicted in Figure 5 was calculated by sampling 18 tRNA (the number of tRNA in the phage genome) with replacement from the host genome.