Adaptive loss of tRNA gene expression leads to phage resistance in a marine cyanobacterium

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Abstract

Bacterial resistance against phage infection is a topic of significant interest, yet little is known for ecologically relevant systems. Here we investigate the mechanisms underlying intracellular resistance of marine *Synechococcu*s WH5701 to the Syn9 cyanophage. Despite possessing restriction-modification and Gabija defense systems, neither contributed to resistance. Instead, resistance was primarily driven by insufficient levels of the Leu^{TAA} tRNA, preventing translation of key phage genes. This resulted from a dramatic reduction in transcripts of both cellular and phage encoded Leu^{TAA} tRNAs. Restoring cellular tRNA expression rendered the cyanobacterium sensitive to infection. This study revealed a passive intracellular mode of resistance, that is distinct from currently known active innate and adaptive defense systems, demonstrating their importance for bacteria-phage interactions. We propose an evolutionary scenario whereby changes in cell codon usage, acquisition of tRNAs by the phage and loss of cell and phage tRNA expression resulted in an effective means of cyanophage resistance, highlighting the dynamic interplay between bacteria and phages in shaping their co-evolutionary trajectories.

Introduction

Bacterial resistance mechanisms against phage infection have been extensively studied over the last several decades. This interest stems from an understanding that phages play a major role in the ecology and evolution of bacterial populations[1,2]. In recent years, the focus on studying phage resistance has increased as the threat of antimicrobial resistance is leading to a resurgence in the use of phages as therapeutics (phage therapy), where resistance to phages pose one of the major hurdles for clinical use[3]. Furthermore, bacterial defense systems, such as restriction-modification (R-M) and CRISPR–Cas systems, have been adapted as important tools for biotechnology. However, experimentation to elucidate molecular mechanisms of resistance in ecologically relevant systems is rare.

Studies of resistance commonly focus on finding genes that confer some degree of protection against infection[3–6]. However, phage resistance is not exclusively due to these types of "active" innate and acquired defense systems. Loss of function, or "passive" modes of resistance, can also protect the host against phage infection. The most well studied examples of this latter type of resistance are mutations that lead to changes in the cell wall, for example, alterations in receptors that impact phage adsorption[7–11], or loss of extracellular polymers needed for adsorption[12,13]. Intracellular resistance caused by a loss of function has been less well studied. Viruses often use intracellular components of the host for their replication and there is evidence that phage infections can fail when these are artificially deleted[14–16]. Yet, there is little evidence that bacteria have naturally evolved resistance to viruses by changes in intracellular components that lead to blocking phage infection inside the cell[17].

Marine picocyanobacteria from the genera *Synechococcus* and *Prochlorococcus* contribute significantly to global primary production[18] . These cyanobacteria coexist with high numbers

of cyanophages in the oceans[19]. In some regions cyanophages can be a substantial cause of mortality, impacting cyanobacterial distribution, primary production and ocean biogeochemistry[20–23]. In other regions low levels of infection ensue[23,24] and coexistence is considered likely to be due to a high degree of cyanobacterial resistance to co-occurring cyanophages[7,19,25].

Previously, we reported that resistance to generalist cyanophages (those with broad host ranges) often acts intracellularly, after adsorption and entry[17]. Syn9 is a marine T4-like cyanomyovirus, with a 177.3 Kb genome which encodes for 226 putative proteins and six tRNAs and has a GC content of 40.5%[26]. Syn9 is a generalist cyanophage that infects multiple strains of *Synechococcus*[17,19,27]. One such sensitive *Synechococcus* strain is the open ocean, phycoerythrin-containing, *Synechococcus* sp. strain WH8102, which belongs to clade III of the *Synechococcus* subcluster 5.1[28,29]. This cyanobacterium has a 2.43 Mb genome that codes for 2,583 proteins with a GC content of 59.4%[30]. It has no currently recognizable defense systems against phages[17], and is sensitive to many phages[19,31,17]. However, Syn9 cannot replicate on a suite of other cyanobacteria, including the estuarine, phycoerythrin-lacking, *Synechococcus* sp. strain WH5701, which belongs to *Synechococcus* subcluster 5.2[28,29]. This cyanobacterium has a 2.86 Mb genome that codes for 3,129 proteins with a GC content of 66%[30]. Notably, this cyanobacterium is resistant to all phages tested on it[17,19]. Based on sequence homology it has two known resistance mechanisms, a type I R-M and a Gabija system[5]. However, neither of these systems have been tested for their ability to provide resistance to phage infection in *Synechococcus* WH5701.

In this study, our primary objective was to unravel the underlying mechanism of intracellular resistance in *Synechococcus* WH5701 against Syn9 infection. We found that the R-M and

Gabija active defense systems were ineffective in conferring resistance against this phage. Rather, we found that resistance against Syn9 stemmed from insufficient levels of the Leu^{TAA} tRNA. This was due to dramatically reduced production of both the cyanobacterial and cyanophage Leu^{TAA} tRNA genes. Our findings underscore the critical role of tRNA availability in phage susceptibility. Furthermore, we revealed a passive form of resistance where the adaptive loss of function of a host component resulted in intracellular resistance to phage infection.

Results

We used two approaches to investigate the mechanism for resistance to Syn9 infection in *Synechococcus* sp. strain WH5701 (also referred to as *Synechococcus* WH5701 or WH5701-WT-Resistant). First, we examined the functionality of the two known defense systems encoded in the genome of *Synechococcus* WH5701. Second, we used the stage at which infection is arrested to help discern the nature of the mechanism underlying resistance. Our previous observations revealed that Syn9 enters the cell, replicates its DNA, translates many but not all its proteins, and begins to assemble capsids[17]. Untranslated proteins include the neck protein, the tail sheath stabilizer, a packaging protein and a protease involved in capsid maturation[17]. Infection stalls at the stage of DNA packaging. Thus, Syn9 phage infection is arrested at a late stage during infection of *Synechococcus* WH5701. In this study, we often compare the infection process of Syn9 in the resistant strain and its derivatives to infection on the sensitive *Synechococcus* sp. strain WH8102 (also referred to as *Synechococcus* WH8102 or WH8102-WT-Sensitive).

Investigating the role of R-M and Gabija systems

We began by examining the two known defense systems present in the genome of *Synechococcus* WH5701. Note that Syn9 DNA degradation during infection of *Synechococcus* WH5701 occurs many hours after the anticipated end of infection based on sensitive strains[17], and is thus different to the rapid degradation expected if DNA targeting systems like R-M and Gabija are responsible for resistance[4,32]. Nonetheless, we still assessed if these systems are effective against the Syn9 phage. We inactivated an essential gene within each system in two separate mutants; the predicted nuclease in the R-M system, *hsdR*[33], and the predicted helicase in the Gabija system, *gajB*[32] (Fig. 1a). If either of these systems were responsible for conferring resistance to the Syn9 phage, gene inactivation would lead to phage sensitivity.

Upon challenging the *Synechococcus* WH5701 inactivation mutants with Syn9 we found no discernible decline in growth of the cyanobacterium (Fig. 1b). Furthermore, no phage plaques were formed (data not shown). In addition, we challenged these mutants with two other T4-like cyanophages that the wild-type (WT) cyanobacterium is resistant to but that can attach and enter the cell[17], S-TIM4 and P-TIM40. Growth of the mutants was not impacted by the S-TIM4 phage (Extended Data Fig.1a) indicating that the R-M and Gabija systems do not confer resistance to this cyanophage either. However, the growth of the mutants was somewhat reduced by the P-TIM40 cyanophage (Extended Data Fig.1b), suggesting these systems provide some degree of protection against this phage. Consequently, we conclude that, irrespective of whether these two defense systems are active against other cyanophages, neither is responsible for resistance to the Syn9 phage. These findings support our previous hypothesis that nuclease-based resistance mechanisms are not responsible for *Synechococcus* WH5701 resistance to Syn9 infection.

Translational inhibition by tRNA availability

We turned to assess whether a novel mechanism is involved in phage resistance. Based on the resistance phenotype of Syn9 during infection of *Synechococcus* WH5701, we hypothesized that resistance is due to the absence of crucial proteins required for DNA packaging, tail attachment, and phage morphogenesis[17]. This could be linked to a failure in their translation, or to their degradation subsequent to translation.

We began by addressing the possibility that the missing proteins are not translated. We hypothesized that a lack of translation of these proteins is due to a mismatch between the codons found in the missing proteins and the tRNA pool in the cell. Hence, we compared codon usage between the 81 phage proteins that were detected in *Synechococcus* WH5701 to the 35 proteins that were missing (of those detected in the sensitive strain by proteomics)[17]. We found that three codons were significantly more prevalent in the non-detected proteins: UUA for Leu^{TAA}, AGA for Arg^{TCT}, and ACA for Thr^{TGT} (Table 1). Notably, two of these codons, UUA and ACA, are rare in the genome of the resistant *Synechococcus* WH5701 relative to other codons (Table 1, Table S1, Extended Data Table 1).

Next, we wondered whether the resistant *Synechococcus* WH5701 carries the genes for these three tRNAs. We found genes for all three of these tRNAs in the genome of this cyanobacterium. We then asked whether insufficient tRNA availability in *Synechococcus* could be preventing translation of the missing phage proteins. We used reverse transcriptase quantitative PCR (RT-qPCR) to measure tRNAs levels in uninfected *Synechococcus* WH5701-WT-Resistant and compared them to those in the sensitive *Synechococcus* WH8102. We found that two of the three tRNAs (Leu^{TAA} and Arg^{TCT}) were below the limit of detection.

This is more than 4 orders of magnitude below tRNA levels for common codons in this cyanobacterium and for these same tRNAs in the sensitive *Synechococcus* WH8102 (Fig. 2a). The third tRNA (Thr^{TGT}), however, was found at high levels in the resistant cyanobacterium despite the corresponding codon being rare in this strain. Interestingly, a fourth tRNA that is relatively rare in *Synechococcus* WH5701, Val^{TAC} (Extended Data Table 1), was also below detection limits, even though its codon is not differentially distributed in present and absent Syn9 phage proteins (Table 1). Thus, despite the presence of the relevant tRNA genes in the genome of *Synechococcus* WH5701, the tRNA levels of some of them were undetectable. Note that this was in uninfected cells and is thus irrespective of the presence of the cyanophage.

Phages often carry tRNA genes, including Syn9, which are thought to compensate for the mismatch between their codon usage and the tRNA pool of the host[26,34–37] or for depletion of host tRNAs[37,38]. Interestingly, four of the six tRNA genes carried in the Syn9 genome correspond to the codons discussed above that are rare (<1 %) or very rare (< 0.5 %)[39] in the genome of resistant *Synechococcus* WH5701 (Extended Data Table 1, Extended Data Fig. 2). This is especially so for UAA which accounts for only 0.09 % of *Synechococcus* WH5701 codons. Furthermore, these tRNA genes match codons common in the Syn9 genome[26,35] (Extended Data Table 1, Extended Data Fig. 2). Therefore, we wondered whether these phage tRNAs were expressed in the resistant cyanobacterium.

We found detectable levels of all six phage tRNAs during infection, but these were approximately 1000-fold lower in the resistant *Synechococcus* WH5701 relative to the sensitive *Synechococcus* WH8102 (Fig. 2b). These findings suggest that the phage tRNAs

were not sufficiently transcribed or did not accumulate in the resistant cyanobacterium, and would not compensate for the low levels resulting from the cyanobacterial genome.

Given the overall low levels of these tRNAs, we hypothesized that *Synechococcus* WH5701 is resistant to Syn9 because the tRNAs required to translate key phage proteins are not present at sufficient levels. This could be due to insufficient tRNA transcription or rapid degradation after transcription. If there is no, or low, transcription then expressing the tRNAs using appropriate regulatory elements would result in tRNA accumulation in the cells. Conversely, if the tRNAs are targeted for degradation post transcription, then additional regulatory elements for transcription would not result in an accumulation of the tRNAs. To test this, we expressed two undetectable WH5701-WT-Resistant tRNA genes, Leu^{TAA} and Arg^{TCT}, together from a replicative plasmid under the control of the cyanobacterial *rnpB* promoter (Fig. 3). Our insert included the sequence coding for the tRNA itself together with the native flanking regions since these can influence tRNA maturation[40,41]. We found that the two tRNAs expressed from the vector were present at levels that were at least 4-orders of magnitude above those in the resistant wild-type *Synechoccocus* WH5701 (Fig. 3a, b). These findings indicate that the tRNAs were not targeted for degradation post-transcriptionally in this expression strain, even though they still maintained their native flanks. Thus, we consider a lack of transcription to more likely be the cause of the absence of these tRNAs in the WH5701-WT-Resistant strain than post-transcriptional degradation.

To test if the presence of these tRNAs was sufficient to allow infection, we exposed the expression strain to the Syn9 cyanophage. We observed a clear decline in growth of the expression strain, similar to that for the WH8102-WT-Sensitive strain and different to the WH5701-WT-Resitant strain (Fig. 4). Furthermore, plaques were formed on lawns of the

expression strain (Fig. 4c). These findings unequivocally show that expression of these two tRNAs resulted in phage sensitivity and that *Synechococcus* WH5701 resistance to Syn9 is due, at least partially, to insufficient levels of specific tRNAs. Since this resulted from transcription of the Leu^{TAA} and Arg^{TCT} tRNAs from the cyanobacterial *rnpB* promoter with no other changes to the tRNA genes nor their flanking regions, these findings suggest that neither specific protein degradation nor specific tRNA degradation are the mechanism of resistance.

Investigating transcriptional loss of tRNAs in Synechococcus *WH5701*

We set out to understand what might be causing the lack of expression of the cyanobacterial tRNA genes. We hypothesized that these genes have lost necessary regulatory motifs for their production. To assess this possibility, we started by cloning the two tRNA genes, Leu^{TAA} and Arg^{TCT}, with new flanking regions, replacing these regions with the flanking regions of the same tRNA genes from the WH8102-WT-Sensitive strain. We also replaced the flanking regions with those of a tRNA found to have high transcript levels, Tyr^{GTA}, from the WH5701-WT-Resistant strain (see tRNA levels in Fig. 2a). These inserts were not cloned with an external promoter to assess whether the flanking regions themselves contained the necessary information for tRNA production (see Fig. 3d for an overview of the different constructs). The levels of both tRNAs were present in high amounts in both *Synechococcus* WH5701 strains (Fig. 3a, b). Thus, these flanking regions were sufficient for tRNA production and resulted in relatively high levels of tRNAs.

Next, we looked for motifs upstream of tRNAs that could be responsible for production. This was done for the seven tRNAs in WH5701-WT-Resistant and the ten tRNAs in WH8102-WT-Sensitive for which high transcript levels was experimentally verified by RT-qPCR (Fig. 2a). We found a conserved motif, TXXG, 35 nt upstream of the first tRNA nt in 14 of the 17

transcribed tRNAs (Fig. 3e). This motif was absent, however, in the same position for the three undetected tRNAs, Leu^{TAA}, Arg^{TCT}, and Val^{TAC} in the WH5701-RT-Resistant strain (Fig. 3e). To test if this motif was needed for tRNA production, we replaced it with AAAA nucleotides in the upstream region of the inserts with the Tyr^{GTA} flanking regions. Indeed, the tRNAs were not detected when the TXXG was removed (Fig. 3a, b). However, tRNA levels did not increase when we added this motif to the native Leu^{TAA} and Arg^{TCT} tRNA genes in the -35 position (Fig. 3a, b). These findings indicate that the TXXG motif is needed but not sufficient for production of these two tRNAs. They further suggest that other regulatory elements in the tRNA flanking regions are necessary for tRNA production, although we did not find evidence of other conserved motifs.

We then assessed whether tRNA production with and without this -35 motif affected sensitivity to Syn9. We challenged the various *Synechococcus* WH5701 plasmid-containing strains with Syn9 and found that high levels of the tRNAs resulted in sensitivity to Syn9 (Fig. 3a, b, c), with plaques being produced on lawns as well as a clear decline in growth of the cyanobacteria (Fig. 5a, b). For the strains in which tRNA transcripts were not observed, no sensitivity to the phage was found (Fig. 3a, b, c), with neither a decline in cyanobacterial growth nor plaque formation resulting (Fig. 5c, d). These findings indicate that tRNA production is sufficient to render *Synechococcus* WH5701 sensitive to Syn9 infection. These same WH5701 tRNA plasmid-containing strains, however, did not become sensitive to infection by the S-TIM4 or P-TIM40 phages, indicating that this *Synechococcus* strain has additional means of resistance against other cyanophages.

As described above, all three *Synechococcus* WH5701 plasmid-containing strains that resulted in detectable tRNA levels were also sensitive to Syn9. Therefore, we wondered whether

phage production is directly related to tRNA transcript levels. To assess this, we cloned two copies of the two tRNA genes (Leu^{TAA} and Arg^{TCT}), each set with its own Tyr^{GTA} flanking regions, into *Synechococcus* WH5701 (Fig. 3d). We observed about a 2-fold increase in transcript levels (Fig. 3a, b), but there was no effect on phage production (Fig. 3c, p=0.7, two tailed U test for EOP of strains with single and double gene copies). Thus, tRNA transcript levels do not correlate directly with phage production, suggesting that other factors also affect phage production.

We were interested in determining whether both tRNAs were required for the sensitive phenotype. We introduced each tRNA separately with the Tyr^{GTA} flanking regions and saw that high levels of Leu^{TAA} alone was sufficient to render *Synechococcus* WH5701 sensitive to Syn9 (Fig. 5e). In contrast, high levels of Arg^{TCT} alone left the cyanobacterium resistant to the Syn9 phage (Fig. 5f). However, phage production was approximately double when both tRNAs were at high levels than just for Leu^{TAA}, with an efficiency of plating of 18% with Leu^{TAA} alone versus 35% with both tRNAs (p=0.05, one tailed U test, Fig. 3c). Hence while high levels of Arg^{TCT} alone did not result in phage sensitivity, this tRNA contributed significantly to phage production.

Finally, we assessed whether production of the tRNA gene for Val^{TAC} also renders *Synechococcus* WH5701 sensitive to infection, since it is also below the limit of detection in this cyanobacterium. For this we cloned the Val^{TAC} with the Tyr^{GTA} flanking regions from the WH5701-WT-Resistant strain, both alone and together with the tRNA genes for Leu^{TAA} and Arg^{TCT} (Fig. 3d). We found that introduction of the Val^{TAC} gene alone did not lead to a reduction in cyanobacterial growth or the production of infective phage (Extended Data Fig. 3a). Its introduction with the other two tRNA genes resulted in sensitivity to Syn9 infection

(Extended Data Fig. 3b), which can be ascribed to Leu^{TAA} production. However, the introduction of all three tRNA genes did not increase phage progeny production beyond that of the other two tRNA genes without Val^{TAC} (Fig. 3c). Thus, despite the lack of detection of this tRNA in the WH5701-WT-Resistant, its introduction did not render *Synechococcus* WH5701 sensitive to infection nor increase phage progeny yield. Since, the Val^{TAC} codon is not differentially distributed in the translated and untranslated Syn9 proteins during infection of the resistant *Synechococcus* WH5701 (Table 1), these findings are consistent with the conclusion that it is the discrepancy in codon usage of select untranslated proteins that is behind the resistance of *Synechococcus* WH5701 to Syn9.

Discussion

Our findings indicate that the lack of production of the Leu^{TAA} gene in *Synechococcus* WH5701 confers it with resistance to infection by Syn9. This was coupled with low transcript levels of the corresponding tRNA carried by the phage. Together the scarcity of Leu^{TAA} tRNAs resulted in the inability of the phage to translate all of its essential proteins[17]. The lack of transcripts of the host tRNA was due, at least partially, to the absence of a motif in the flanking region of the tRNA gene (this study), while the mechanism for the decline in phage tRNA transcripts (together with other Syn9 phage genes[17]) remains unknown. The absence of host tRNAs can be considered a passive mode of resistance since it is caused by the loss of expression of a component in the cell that is required by the phage and which does not affect the growth of the cell[17]. This mode of resistance acts at a late stage of infection, since Syn9 gets as far as building capsids[17]. Thus, resistance is not the result of early stage incompatibility between this cyanophage and cyanobacterium.

The most well-known passive modes of resistance in bacteria arise from changes in an outer cellular component that phages use as receptors, preventing phage adsorption and entry into the cell[6,7–11], and thus function extracellularly. Intracellular mutations or loss-of-function of cellular components as a means of resistance is rarely reported in bacteria, but based on our findings, may be more common than currently appreciated. For example, we previously reported that many marine cyanophages enter into marine cyanobacteria but cannot complete their infection cycle[17]. Most of these cyanobacteria (12 out of 16 marine *Synechococcus* and *Prochlorococcus* strains) lack recognizable active mechanisms of resistance[17]. At this stage, it remains unknown whether the means of resistance in these cells are passive, similar to that reported here, or whether they carry currently unknown active defense systems. Intriguingly, one of these strains, *Synechococcus* RS9917 (belonging to *Synechococcus* subcluster 5.1, clade VIII)[29,30], displays intracellular resistance to Syn9 at an early stage of infection[17] and has severe mismatches to Syn9 codons even for early genes (Extended Data Table 1, Extended Data Fig. 2). Furthermore, Syn9 transcription is low overall in *Synechococcus* RS9917[17], similar to *Synechococcus* WH5701, likely preventing compensation by the phage-encoded tRNAs. Interestingly, *Synechococcus* RS9917 and WH5701 also have an unusually high number of genes in common for their phylogenetic distance[30]. However, since codon usage discrepancy alone is insufficient to ascertain whether a cell type will be resistant or sensitive to a phage (Extended Data Fig. 2), further work is needed to determine if the same or a different mode of resistance is in play in the *Synechococcus* RS9917-Syn9 interaction.

Recent research has uncovered a plethora of new systems of active innate and acquired defense systems in bacteria[42,5,43,6]. However, little is known about potential passive intracellular modes of resistance for bacteria-at-large. Thus, further investigation into this

phenomenon has the potential to significantly enhance our understanding of phage-bacteria interactions and the evolutionary dynamics underlying bacterial resistance to phages.

Active defense systems that target translation have been demonstrated previously against phages. Such systems cleave essential host translation factors[44] or target tRNAs for degradation[45]. These are abortive infection systems that result in the death of the infected cell and provide protection to the population by preventing the spread of the phage[46]. This is in stark contrast to the means of resistance reported here which is both passive and does not result in the death of the cyanobacterium. Thus, it is now clear that both active, abortive, defense systems and passive, non-abortive, modes of resistance work at the level of translation inhibition.

Recently, it has been proposed that some phages carry their own set of tRNA genes to replenish the pool of host tRNAs degraded by active defense system or that are depleted due to degradation of the host genome[37,38]. In these interactions host tRNA loss is induced by phage infection. In our current interaction, however, the lack of host tRNA transcripts is constitutive (Fig. 2a), rather than being induced by the presence of the phage or other stressors[37,47,48]. This argues against an active phage defense system that degrades host tRNAs. Furthermore, the 1000-fold increase in tRNA levels upon addition of the *rnpB* transcriptional regulatory element without any change to the tRNA gene or its flanking regions (Fig. 3) also indicates that a tRNA degradation defense system is not in play, especially since all known sites for tRNA degradation are found in the sequence of the tRNA itself[47,49–52].

The lack of proper tRNA maturation can result from changes in tRNA flanking regions, leading to their degradation[53]. Thus, it is feasible that de novo transcription or appropriate

tRNA maturation of the Leu^{TAA} tRNA is responsible for tRNA accumulation and Syn9 sensitivity of *Synechococcus* WH5701 upon introduction of flanking regions from other expressed tRNAs and the TXXG motif therein (Fig. 3). While we can't rule out the lack of proper maturation as a means for tRNA depletion, we consider it to be less likely than the lack of transcription. This is because defective tRNAs would be detectable at some level upon constant transcription and turnover[41,50], in contrast to our findings for WH5701-WT-Resistant (Fig. 2a). Furthermore, we observed accumulation of tRNAs and sensitivity to Syn9 upon expression from the *rnpB* cyanobacterial promoter that has native WH5701-WT-Resistant Leu^{TAA} flanks (Fig. 4). Thus, with the evidence currently at hand, we consider a lack of host tRNA transcription to be the more parsimonious explanation for the absence of cyanobacterial tRNAs and resistance to Syn9 in *Synechococcus* WH5701.

In this study we focused our work on investigation of the transcript levels of the cyanobacterial tRNAs. While restoring expression of the leucine and arginine tRNAs was sufficient to result in phage sensitivity in *Synechococcus* WH5701, phage production was still considerably lower than in the naturally sensitive *Synechococcus* WH8102 strain. This may, in part, be due to the orders of magnitude lower expression of all six of the phage tRNAs, including the phage leucine and arginine tRNAs (Fig. 2b). It could also be due to low transcription of many Syn9 transcript in this resistant strain, ~10-110% of that in the sensitive strain[17]. This disparity in tRNA and mRNA transcript levels could stem from low transcriptional compatibility of Syn9 to the resistant *Synechococcus* WH5701. However, it is also possible that this lower efficacy of transcription is the result of a currently unknown active defense system, which could be coded for by one of the many unique genes in this resistant *Synechococcus* strain[30]. The two known active defense systems in the genome of *Synechococcus* WH5701 could confer resistance against other phages, as may the lack of

transcription of the Val^{TAC} tRNA (Fig. 2a). Currently, no known phage can successfully infect *Synechococcus* WH5701[17,19], and the introduction of the Leu^{TAA} tRNA did not render this strain sensitive to two other cyanophages. Therefore, it appears that this *Synechococcus* strain has evolved to possess multiple layers of defense that protect it against a wealth of cyanophages, culminating in the emergence of a cyanobacterium that is quite a phage-resistant specialist.

The evolution of the lack of production of certain tRNA genes in *Synechococcus* WH5701 is likely to have occurred gradually. Clearly, a phage's ability to translate its proteins and successfully infect a host is dependent on its ability to utilize the tRNA pool of the host[54,55]. Since lytic phages kill off the cell, it is considered likely that phages exert selective pressure on the host that influences its codon usage pattern and leads to differentiation from that of the phage[36,55,56]. Our study provides experimental evidence in support of this hypothesis.

Combining our data with theoretical evidence from the literature, we propose a multi-step scenario that can explain the evolutionary process that led to the loss of tRNA transcripts as an effective means of cyanophage resistance in *Synechococcus* WH5701 and perhaps other bacteria. Phages and other parasites often have low GC content which is potentially due to the lower cost of producing AT rich genomes and the higher availability of these nucleotides[57]. We hypothesize that the cyanobacterium underwent adaptations that modify its codon usage patterns to differ from those most critical for the expression of phage replication and morphogenesis genes[34,54,36]. This is likely to have preceded, or occurred simultaneously with the downregulation of tRNA gene expression[58–60,51] through the loss of at least the TXXG motif at the -35 position of three key tRNA genes (Fig. 2a). These tRNAs are among those that match the most common codons in T4-like cyanophage genomes[35,36] and thus their lack

of expression is likely to have conferred resistance against infection by T4-like cyanophages, such as Syn9. This deficiency would exert substantial selective pressure for the acquisition and expression of the relevant tRNA genes by cyanophages, to compensate for such codon usage discrepancies[34,36] and allow for infection. Subsequently, we propose that *Synechococcus* WH5701 developed a presently unknown mechanism to limit transcription of cyanophage tRNA genes (as well as other Syn9 genes[17]), such that their presence was rendered ineffectual, preventing infection. Thus, in this scenario, the evolution of resistance in this cyanobacterium stemmed from the gradual loss of tRNA gene expression, whether encoded by the cell or those acquired by the phage, underscoring the dynamic interplay between bacterial hosts and phages in shaping their co-evolutionary trajectories.

Methods

Cyanobacterial growth and cyanophage propagation

Synechococcus WH5701 and WH8102 cultures were grown in an artificial seawater medium (ASW)[17]. Cultures were grown at 21°C at a light intensity of 15-25 μmol photons·m^{-2}·s^{-1} under a 14:10 light:dark regime. Culture growth was measured using chlorophyll *a* autofluorescence (excitation/emission: 440/680 nm) as a proxy for biomass using a Synergy 2 microplate reader (BioTek). Under these conditions, the growth rates of these strains are 2.5 and 1.5 days for *Synechococcus* WH5701 and WH8102 respectively.

For isolation of conjugated colonies, pour plating was used, the cyanobacterial culture was serially diluted and mixed with ultra-pure low-melting point agarose (Invitrogen) at 0.28% in growth medium which was also supplemented with 1 mM sodium sulfite. The helper bacterium, *Alteromonas sp.* strain EZ55, was added to the plates to obtain high plating efficiency[61].

The Syn9 cyanophage was propagated by infecting a sensitive strain at a low multiplicity of infection (MOI) of <0.01 and allowing the culture to clear. Cells were removed from the phage lysates by filtration through a 0.2 μm pore-sized filter. For small volumes (< 20 ml) Acrodisc syringe filters (PALL) were used; for large volumes Nalgene Rapid Flow 50 mm filter units (Thermo Scientific) were used.

The titer of the lysate (the concentration of infective phages) was determined by plaque assay. The phage lysate was serially diluted and mixed with the sensitive *Synechococcus* strain WH8102 at 2×10^6 cells per plate in pour plates (as described above, but without the addition of the EZ55 helper bacterium) to form a cyanobacterial lawn on which clearings in the lawn (plaques) could be counted. The plates were incubated at cyanobacterial growth conditions. Plaques were counted until no new plaques appeared. Images of plates with plaques presented here were adjusted for contrast and brightness using the Microsoft Photos application. Adjustments were made across the entire image equally for all images.

Insertional inactivation of Synechococcus *WH5701 genes*

The Type I restriction system was inactivated by disrupting the restriction subunit gene (*hdsR*; protein ID WP_071934184.1), while the Gabija system was inactivated by interrupting the helicase gene (*gajB*; protein ID WP_071934305.1). Gene inactivation was achieved through plasmid integration into the host chromosome via a single homologous recombination event following Brahamsha[62]. A 250 bp homologous segment was modified to include stop codons in all three reading frames to ensure gene inactivation (see Fig. 1a) and was cloned into a pBR322-derived plasmid[62] (named pDS1)[63]. The chloramphenicol resistance gene in this plasmid was codon optimized for expression in cyanobacteria[64]. The plasmid was

electroporated into the S17 *E. coli* donor strain[65] and colonies were selected on LB plates with 100 µg/ml ampicillin. The plasmid was then mobilized into *Synechococcus* WH5701 by conjugation. Colony formation was performed using pour-plating in the presence of 2 µg/ml chloramphenicol. Successful gene interruption was confirmed by PCR using primers within the plasmid and upstream or downstream of the crossover region in the cyanobacterium (see Table S2 for primer sequences). To ensure full segregation (disruption of all copies of the cyanobacterial chromosome) PCR was performed to verify the absence of wild-type gene copies. Disruption of transcription was verified by RT-qPCR using primers flanking the site of recombination (see Table S2 for primer sequences). Further details on RT and qPCR are provided below.

Analyzing host and phage codon usage and motif searches

Codon usage in the genomes of *Synechococcus* WH5701, *Synechococcus* WH8102, and Syn9 was analyzed with the frequency of each of the 61 codons counted. Additionally, for phages, codon usage per gene was assessed, comparing the average occurrence of codons between translated and untranslated phage proteins in *Synechococcus* WH5701, as per Zborowsky and Lindell[17]. Codon usage of Syn9 early, middle and late expression clusters was calculated individually for each cluster. Division of Syn9 genes to expression clusters was done according to Doron et al.[27]. The determination of codon usage was carried out using the Countcodon program version 4 with default parameters for bacteria (https://www.kazusa.or.jp/codon/countcodon.html). Statistical comparisons of codon usage per gene were conducted using the Mann-Whitney U test.

Analysis of codon usage preferences and correlation of codon frequencies was done as previously described by Limor-Waisberg et al.[35] and Enav et al.[36]. Correlation of codon

frequencies between Syn9 gene expression clusters and each cyanobacterium was calculated using either the Pearson or Spearman correlation coefficient. If the codon usage of the host and the phage expression cluster was approximately normally distributed the Pearson correlation coefficient was calculated. Otherwise, we used the Spearman correlation coefficient. Normal distribution of the data was assessed using the Lilliefors test for normality.

The TXXG motif was identified after manual inspection of the upstream region of the 10 tRNA genes in *Synechococcus* WH5701 for which tRNA levels were investigated using RT-qPCR. The upstream regions of the 7 expressed tRNAs were compared to the 3 tRNA genes that were not expressed in this cyanobacterium. The motif logo for this region was generated using the WebLogo online tool (https://weblogo.berkeley.edu/logo.cgi)[66] using the 7 expressed tRNAs from *Synechococcus* WH5701 and the 10 expressed *Synechococcus* WH8102 genes (Fig. 2a).

Expression of tRNA genes in Synechococcus *WH5701*

To explore the impact of the absence of Leu^{TAA} and Arg^{TCT} tRNAs on resistance in *Synechococcus* WH5701, these genes were expressed from a replicative plasmid with different regulatory elements. (1) Native tRNA sequences and flanking regions of the Leu^{TAA} and Arg^{TCT} genes as present in the *Synechococcus* WH5701 genome, with the two tRNA genes cloned downstream of the *rnpB* promoter and a chloramphenicol resistance gene. (2) The same tRNA sequences as in (1) with flanking regions of each gene replaced with the flanking regions of its homolog from the sensitive *Synechococcus* WH8102 in a region of the plasmid without a promoter, and (3) with flanking regions of the Tyr^{GTA} tRNA from *Synechococcus* WH5701, which include the TXXG motif in the -35 position relative to the

first tRNA nucleotide in a region of the plasmid without a promoter. (4) The same as in (3) but with the TXXG motif replaced with AAAA. (5) Native tRNA sequences and flanking regions but with the addition of TXXG to the -35 position. (6) The same as (3) but with two copies of each gene. (7 and 8) The same as in (3) but for only one of the tRNA genes (WH5701 Leu^{TAA} alone Tyr^{GTA} flanks or WH5701 Arg^{TCT} alone Tyr^{GTA} flanks). (9) the Val^{TAC} tRNA gene with the Tyr^{GTA} flanks from *Synechococcus* WH5701. (10) Leu^{TAA}, Arg^{TCT} and Val^{TAC} with the Tyr^{GTA} flanks from *Synechococcus* WH5701. (11) Same as (10) but two gene copies of each gene. Constructs were synthesized and cloned into a pUC57-Amp plasmid (Genewiz) and were then inserted into the pDS-ProCAT plasmid[64] using restriction enzymes. The pDS-ProCAT plasmids were then electroporated into *E. coli* S17 and subsequently conjugated into *Synechococcus* WH5701 as described above. Selection of positive colonies was done as described above, except that *E. coli* S17 colonies harboring the plasmid were selected on LB plates supplemented with 750 μg/ml erythromycin (Em).

Determining transcription levels by reverse transcription

For assessing tRNA levels and the expression of interrupted R-M and Gabija genes, total RNA was extracted from cyanobacterial cells. Samples were taken 5 hours after phage infection, as were non-infected controls, and were centrifuged at 9,000 g for 15 minutes at 4ºC. Cells were immediately flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

For RNA extraction, cells were thawed on ice and resuspended in a solution containing 10 mM Tris-HCl pH 8, 100 units of RNase inhibitor (Applied Biosystems), and 15,000 units of Lysozyme (Sigma). After incubating at 37°C for 30 minutes for initial cell wall degradation, RNA was isolated using the Quick RNA Mini Prep kit (Zymo). To remove residual DNA, the

Turbo DNA Free kit (Invitrogen) was used, incubating with DNase at 37°C for 1 hour. Reverse transcription (RT) was conducted with random hexamer primers using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The RT procedure included primer extension at 25°C for 10 minutes, cDNA synthesis at 37°C for 120 minutes, and reaction termination at 85°C for 5 minutes. Samples were diluted five-fold in 10 mM Tris-HCl pH 8 and stored at -20°C before real-time quantitative PCR. The *Synechococcus* WH5701 *rnpB* gene served as a positive control for reverse transcription and as a standardizing marker for gene expression. No RT controls were performed on all samples to ensure that reported transcript levels did not originate from residual phage DNA.

Real-time quantitative PCR

PCR was conducted using the LightCycler 480 Real-Time PCR System (Roche). The cycling program began with a denaturation step of 95°C for 10 minutes, followed by 35-40 cycles of amplification. Each cycle involved denaturation at 95°C for 10 seconds, annealing at 52°C-68°C for 10 seconds, and elongation at 72°C for 10 seconds. Fluorescence was measured for each reaction (FAM; Ex/Em 465/510nm) at the end of each cycle. The LightCycler 480 software (release 1.5.0) was used to calculate the point at which the fluorescence of a sample exceeded the background fluorescence (Cp) using the absolute quantification/2nd-derivative maximum analysis package. Melting curve analysis on the LightCycler 480 instrument verified the specificity of the amplified PCR product.

Each qPCR reaction contained 1X LightCycler 480 SYBR Green I Master mix (Roche), 200 nM desalted primers, and 5 µl template (in 10 mM Tris-HCl pH8) in a total reaction volume of 20 µl (see Table S2 for primer sequences). The number of DNA copies in the reaction was determined by comparing the Cp to that of a sample with a known DNA concentration using a

standard curve. Genomic DNA for standard curves was extracted using the DNeasy Blood & Tissue kit (Qiagen) from cyanobacterial strains and a phenol:chloroform based method for the phages. DNA concentrations in ng/ml were measured by absorbance at 260 nm using a Synergy 2 microplate reader (BioTek) and converted to gene copies/ml by inputting the genome length of the cyanobacterium or phage into the URI Genomics & Sequencing Center calculator for determining the number of copies.

Determining resistance or sensitivity to phage infection

To assess sensitivity or resistance to each phage, cultures were exposed to phage in liquid medium, and their growth was monitored for 2 weeks. It should be noted that these cyanobacteria have division rates on the order of a doubling every 1.5-2.5 days under the conditions used here and that regrowth consistent with spontaneous mutations that confer resistance appear after approximately a 4-6 weeks in the sensitive *Synechococcus* WH8102 strain. This is well after the period of these experiments.

To maintain consistent phage-bacteria ratios at the beginning of the experiments, cells were quantified using flow cytometry with the Influx flow cytometer (Becton Dickinson), based on forward scatter and autofluorescence (emission at 692/640nm). Yellow-green 1 μm diameter microspheres (Fluoresbrite) served as internal standards for size and fluorescence consistency. Culture growth was monitored using chlorophyll *a* fluorescence (as described above). Furthermore, sensitivity to the phage was tested by plaque assay. A strain was considered sensitive if there was either reduced growth in liquid relative to uninfected controls or visible clearings in plaque assays or deemed resistant if there was no growth reduction in liquid medium and no plaque formation on cyanobacterial lawns.

Statistical analysis

All statistical analyses were performed using the IBM SPSS statistics version 24. Mann-Whitney's U test (nonparametric) was used. Normality was tested using the Shapiro Wilk test for normality. Equality of variance was tested using the Levene's test using SPSS software.

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Author contributions

S.Z. and D.L. conceived the project and designed the experiments. S.Z. carried out the experiments, and S.Z. and R.T. analyzed the data. S.Z. and D.L. wrote the manuscript with input from R.T.

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Table 1. Codon usage and frequency for select tRNAs in *Synechococcus* and Syn9 genomes

Codon	AA	tRNA[a]		Codon frequency in genome (%)[b]			Average number of appearances per gene		p value[c]
		WH5701	Syn9	WH5701	WH8102	Syn9	translated	not translated	
UUA	Leu	TAA	TAA	0.09	0.46	1.80	1.4	3.8	0.0006
AGA	Arg	TCT	TCT	1.31	1.42	2.19	2.6	4.9	0.012
ACA	Thr	TGT	TGT	0.89	1.30	2.29	3.3	4.9	0.049
GUA	Val	TAC	TAC	0.49	0.54	1.64	2.4	4.1	0.59
GCA	Ala	TGC	TGC	0.72	1.23	2.85	4.7	8.2	0.31
AAC	Asn	GTT	GTT	1.08	1.67	1.98	3.6	6.2	0.34
UAC	Tyr	GTA		2.30	0.48	1.11	1.9	3.2	0.55
GGC	Gly	GCC		3.50	2.91	1.11	0.9	4.2	0.14
CGG	Arg	CCG		2.48	2.31	0.25	0.2	0.3	0.106
GCC	Ala	GGC		3.47	2.94	0.42	1.0	1.3	0.19

[a]The ten tRNAs whose levels were measured in Fig. 2a are shown here. See Table S1 for a complete list of codons and their frequencies.

[b]The frequency of each codon is the number of times it appears in the genome relative to all codons.

[c]The number of times a codon appears per gene in Syn9 proteins that were translated (n=81) versus proteins that were not translated (n=35) during infection of *Synechococcus* WH5701 was compared using the Mann Whitney U test.

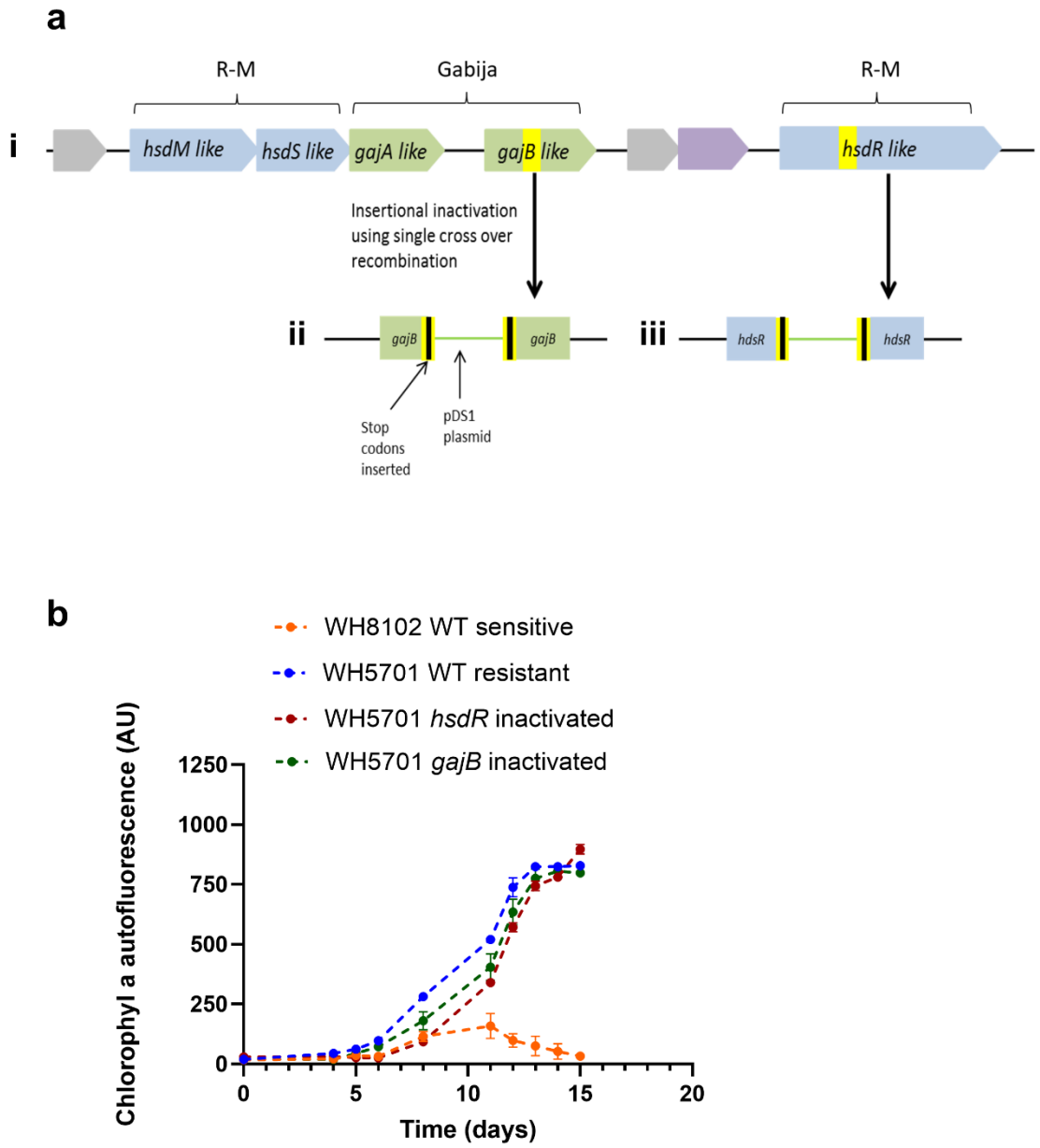


Fig. 1. The effect of known defense system genes on the sensitivity of *Synechococcus* WH5701 to the Syn9 phage. **a** Schematic representation of the organization of the two defense systems in the *Synechococcus* WH5701 genome (i) and in the relevant region in the Gabija system (ii) and R-M system (iii) in the mutants after their insertional inactivation with the pDS1 plasmid (see Methods). **b**. Growth of cyanobacteria in the presence Syn9. Average and standard deviation of 5 biological replicates. In (a), blue shows the R-M type I system, green shows the Gabija system, yellow indicates the homologous regions cloned into pDS1, and black indicates where stop codons were inserted. For the R-M system, *hsdM* encodes the modification subunit, *hsdS* the specificity subunit and *hsdR* the endonuclease. For the Gabija system, *gajA* encodes the endonuclease and *gajB* the helicase. The *gajB* and *hsdR* genes were inactivated. AU=arbitrary units.

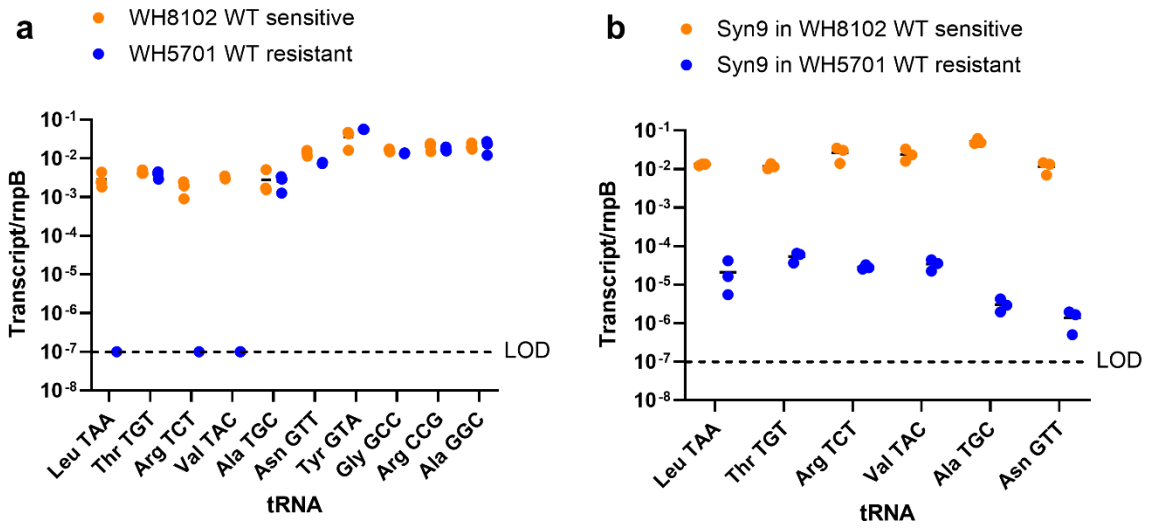


Fig. 2. Phage and cyanobacterial tRNA levels. a. Levels of cyanobacteria-encoded tRNAs during growth (without infection) for the 6 tRNA genes which have homologues in the Syn9 phage (Leu^{TAA}, Thr^{TGT}, Arg^{TCT}, Val^{TAC}, Ala^{TGC}, Asn^{GTT}) and 4 tRNA genes with common codons in the cyanobacterial strains (Tyr^{GTA}, Gly^{GCC}, Arg^{CCG} and Ala^{GGC}). **b**. Levels of the phage-encoded tRNAs during infection of the resistant *Synechococcus* WH5701 WT and sensitive *Synechococcus* WH8102 WT strains, 5 hours after infection with the Syn9 phage. LOD - limit of detection of the RT-qPCR is approximately 100 transcripts. All data is normalized to levels of cellular *rnpB*.

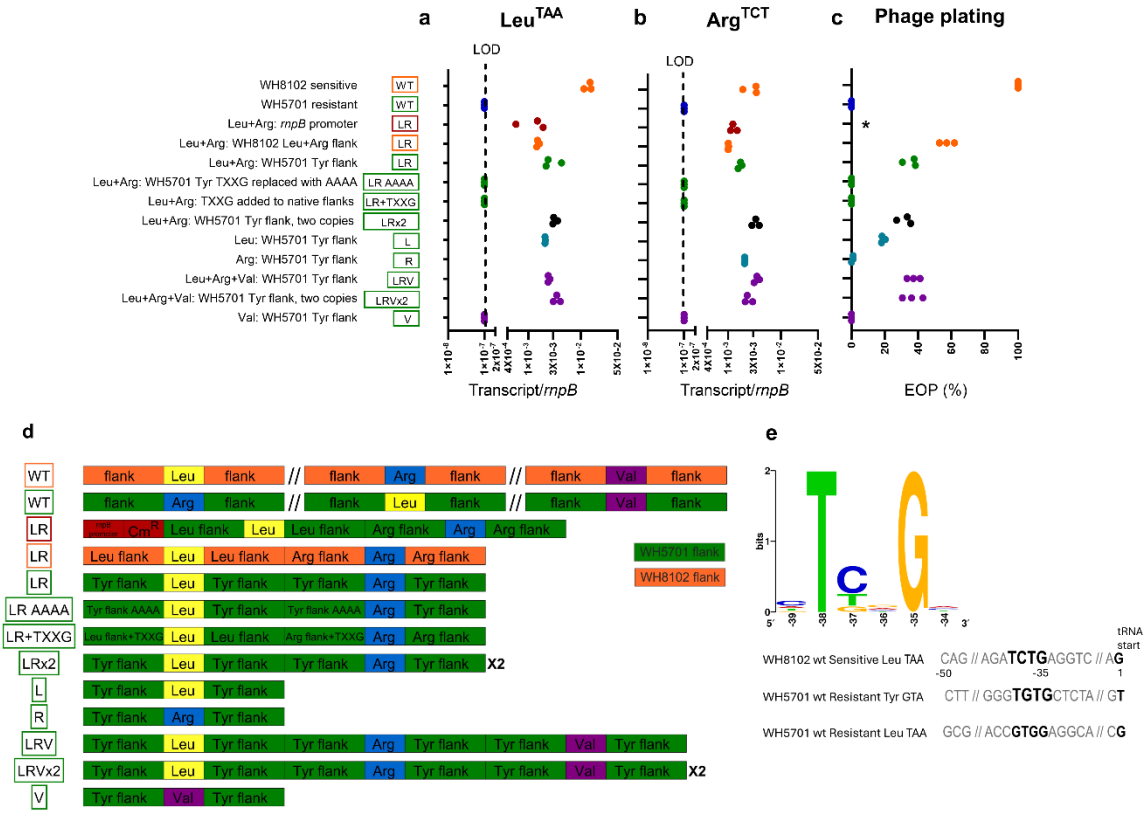


Fig. 3. Expression of Leu^{TAA} and Arg^{TCT} tRNAs from, and efficiency of plating on, expression strains. Transcript levels of the Leu^{TAA} tRNA (**a**) and the Arg^{TCT}gene (**b**), and efficiency of plating (EOP) of Syn9 on expression strains relative to the sensitive *Synechococcus* WH8102 (**c**). Three biological replicates are shown. Colors match those used in Fig. 4 and 5 and Extended Data Fig. 3, where relevant. LOD, limit of detection (10^{-7} transcripts/*rnpB*). * represents that plaques were obtained, but cannot be quantitatively compared to the rest of the strains. (**d**) A schematic representation of the three tRNA genes relevant for this study, Leu^{TAA}, Arg^{TCT}, Val^{TAC}, and their arrangement and flanking regions in the strains used here. The top two WTs represent the tRNAs in the genomes of *Synechococcus* WH8102-WT-sensitive (orange) and *Synechococcus* WH5701-WT-resistant (green), whereas the rest are for the various plasmids introduced into *Synechococcus* WH5701 for construction of the expression strains. The *rnpB* promoter and chloramphenicol resistance gene (Cm^{R}) are in red. Flanking regions from the WH8102-WT-sensitive are in orange, and those from WH5701-WT-resistant are in green. The tRNA gene the flank was taken from appears inside the flank box. Tyr represents Tyr^{GTA}, a tRNA found in high levels in WH5701-WT-resistant. The tRNA genes on the plasmids were always taken from the WH5701-WT-resistant strain (**e**) Logo of the TXXG motif and examples of the upstream region of the Leu^{TAA} tRNA gene in the sensitive *Synechococcus* WH8102 and the Tyr^{GTA} tRNA gene in the resistant *Synechococcus* WH5701 where the motif is present, and the same region in the Leu^{TAA} of the latter strain where the motif is missing.

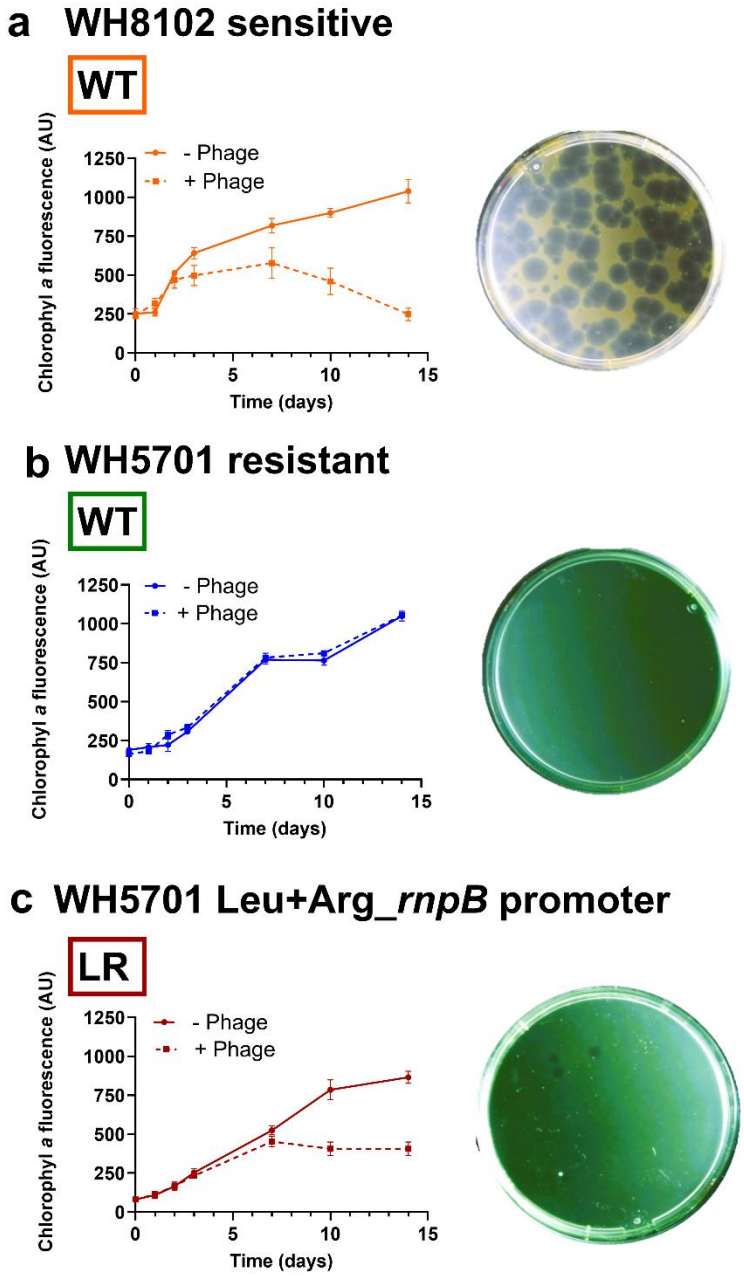


Fig. 4. **Sensitivity of *Synechococcus* WH5701 tRNA expression strain with *rnpB* promoter to infection by Syn9.** Growth of cyanobacterial strains in the presence and absence of Syn9 (left) and plaque formation of Syn9 (right) on sensitive *Synechococcus* WH8102 WT (**a**)**,** resistant *Synechococcus* WH5701 WT (**b**), and WH5701 expression strain where the tRNA genes were cloned under an *rnpB* promoter (**c**). Growth in liquid is average and standard deviation of 5 biological replicates. Plates with Syn9 plaques on cyanobacterial lawns are representative images of 3 biological replicates. WH8102 and WH5701 refer to WT *Synechococcus* WH8102 and WT *Synechococcus* WH5701, respectively. Boxed color-coded abbreviations (WT and LR) match those used in Fig. 3d showing a schematic representation of the arrangement of elements in the *Synechococcus* WT genomes and in the tRNA expression strain with the *rnpB* promoter.

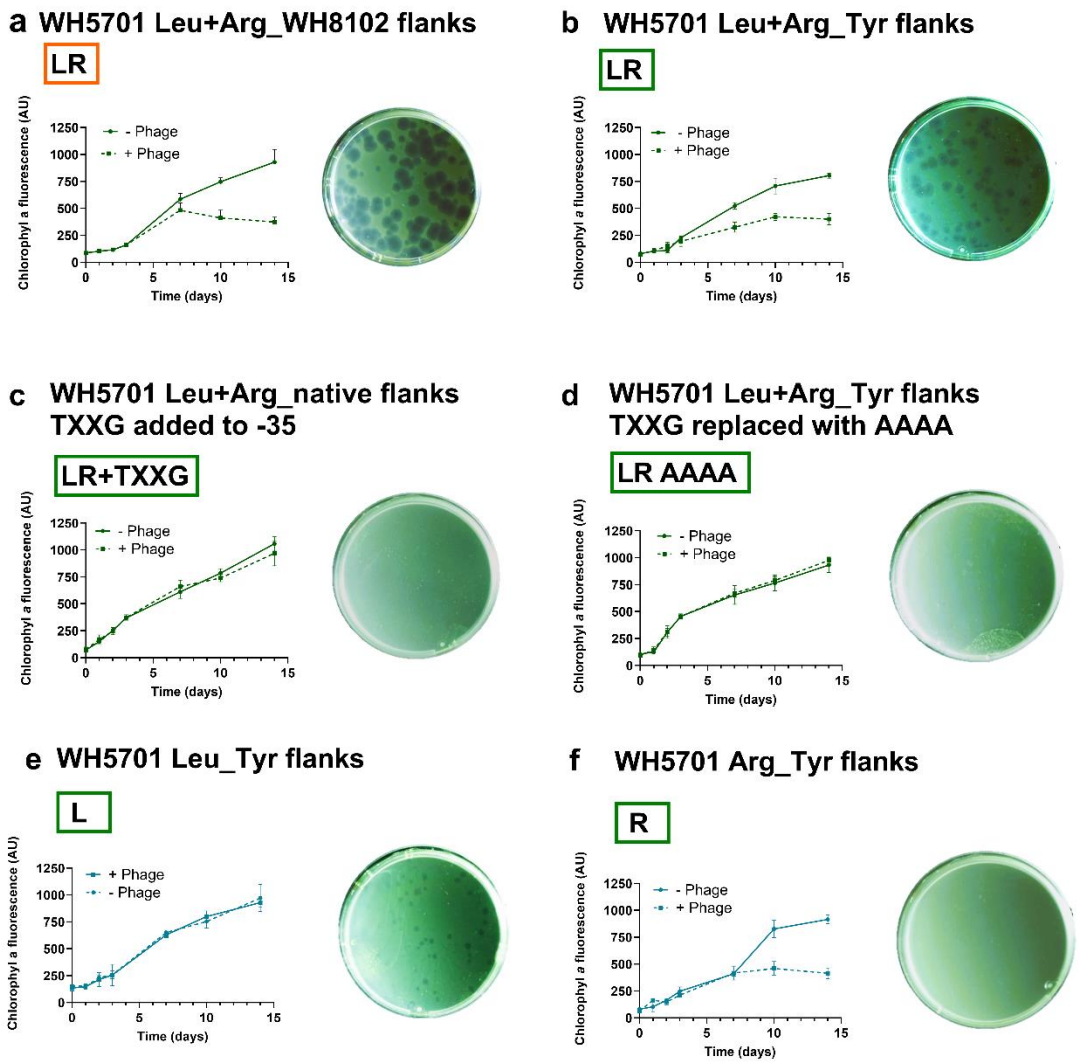


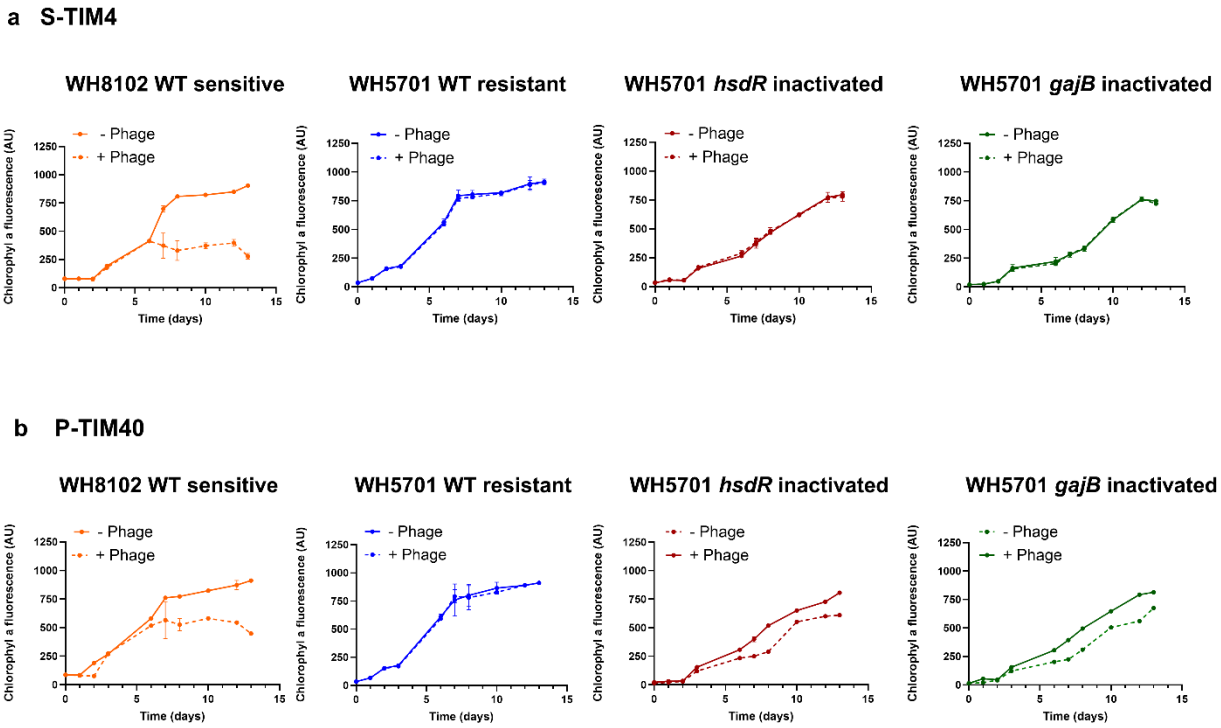
Fig. 5. **Relevance of tRNA flanking regions for tRNA transcription and phage sensitivity.** Growth of cyanobacterial strains in the presence and absence of Syn9 (left) and Syn9 plaque formation (right) on *Synechococcus* WH5701 expression strains where the Leu^{TAA} and Arg^{TCT} tRNA genes were cloned: with the flanking regions of Leu^{TAA} and Arg^{TCT} of *Synechococcus* WH8102 (**a**), the WH5701 Tyr^{GTA} tRNA flanking regions (that naturally contain the TXXG motif at the -35 position relative to the first nucleotide of the tRNA) (**b**), with the Tyr^{GTA} tRNA flanking regions where the TXXG motif was replaced with AAAA (**c**), and with the native Leu^{TAA} and Arg^{TCT} flanking regions where the TXXG motif was inserted at the -35 position (**d**). *Synechococcus* WH5701 expression strain with the Leu^{TAA} tRNA gene alone with Tyr^{TGA} flanking regions (**e**). *Synechococcus* WH5701 expression strain with the Arg^{TCT} tRNA gene alone with Tyr^{TGA} flanking regions (**f**). Growth in liquid is average and standard deviation of 5 biological replicates. Plates with Syn9 plaques on cyanobacterial lawns are representative images of 3 biological replicates. WH8102 and WH5701 refer to *Synechococcus* WH8102 and *Synechococcus* WH5701, respectively. Boxed color-coded abbreviations match those used in Fig. 3d which shows the schematic representation of the arrangement of tRNA genes and flanking regions in the expression strains.

Extended Data Table 1. Codon usage preferences for tRNA genes present in the Syn9 genome, in Syn9 expression classes, and in marine cyanobacteria.

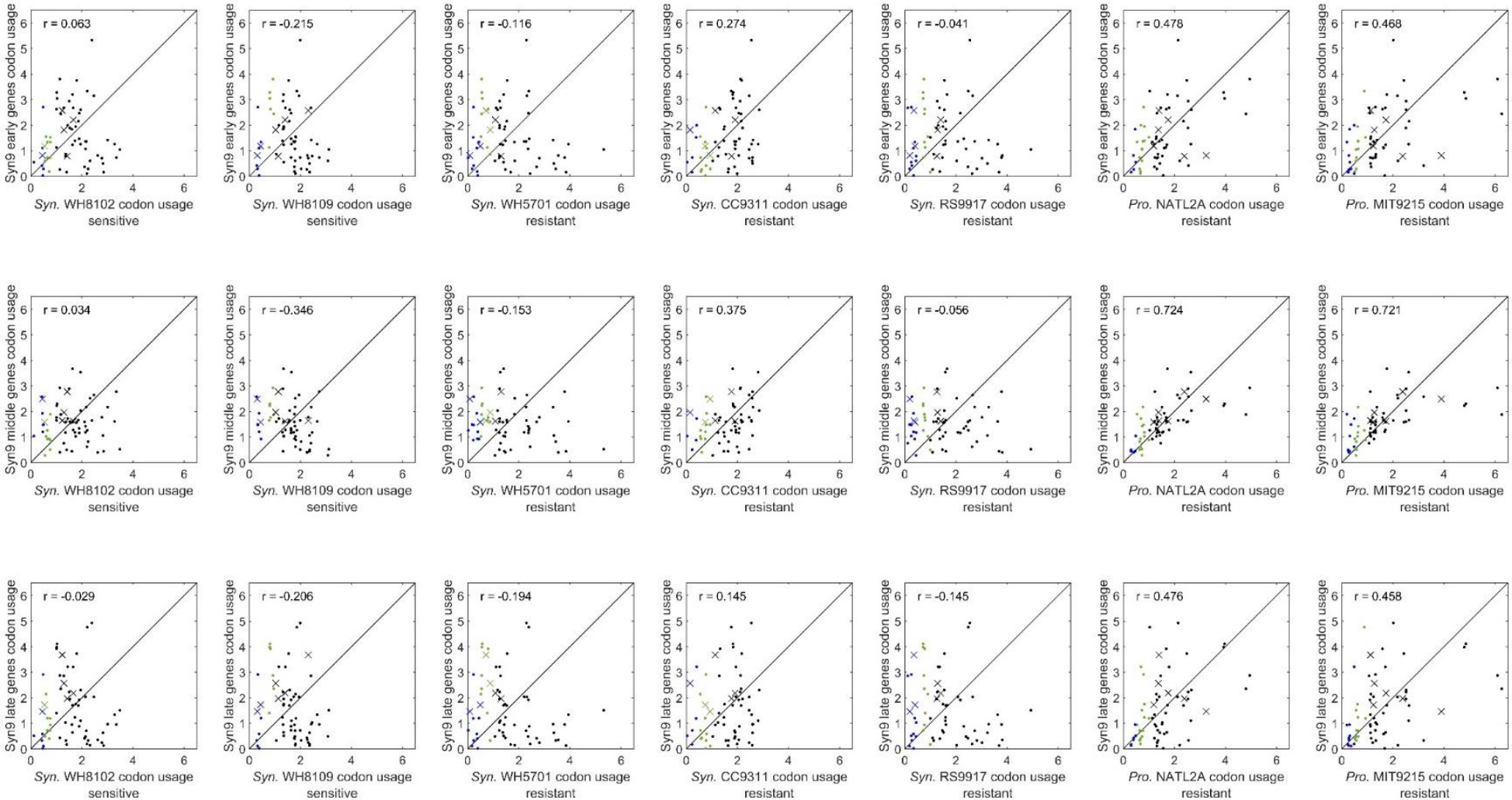
Amino acid	**V**	**T**	**A**	**N**	**L**	**R**
Codon[a] / **Genome**	**GUA**	**ACA**	**GCA**	**AAC**	**UUA**	**AGA**
Syn9 early genes	1.18	1.81	2.57	2.21	0.81	0.79
Syn9 middle genes	1.58	1.96	1.64	1.62	2.49	2.78
Syn9 late genes	1.72	2.57	3.68	2.18	1.46	1.97
Syn9 entire genome	1.64	2.29	2.85	1.98	1.80	2.19
Synechococcus WH8102, Sensitive	0.54	1.30	1.23	1.67	0.46	1.42
Synechococcus WH8109, Sensitive	0.45	1.03	2.31	1.40	0.31	1.14
Synechococcus WH5701, Resistant	0.49	0.89	0.72	1.08	0.09	1.31
Synechococcus CC9311: Resistant	0.74	0.15	1.13	1.91	0.94	1.78
Synechococcus RS9917, Resistant	0.40	1.28	0.36	1.41	0.20	1.26
Prochlorococcus NATL2A, Resistant	1.19	1.38	1.39	1.76	3.24	2.39
Prochlorococcus MIT9215, Resistant	1.24	1.28	1.13	1.73	3.90	2.38

[a] The frequency of each codon is the number of times it appears in the genome relative to all codons.

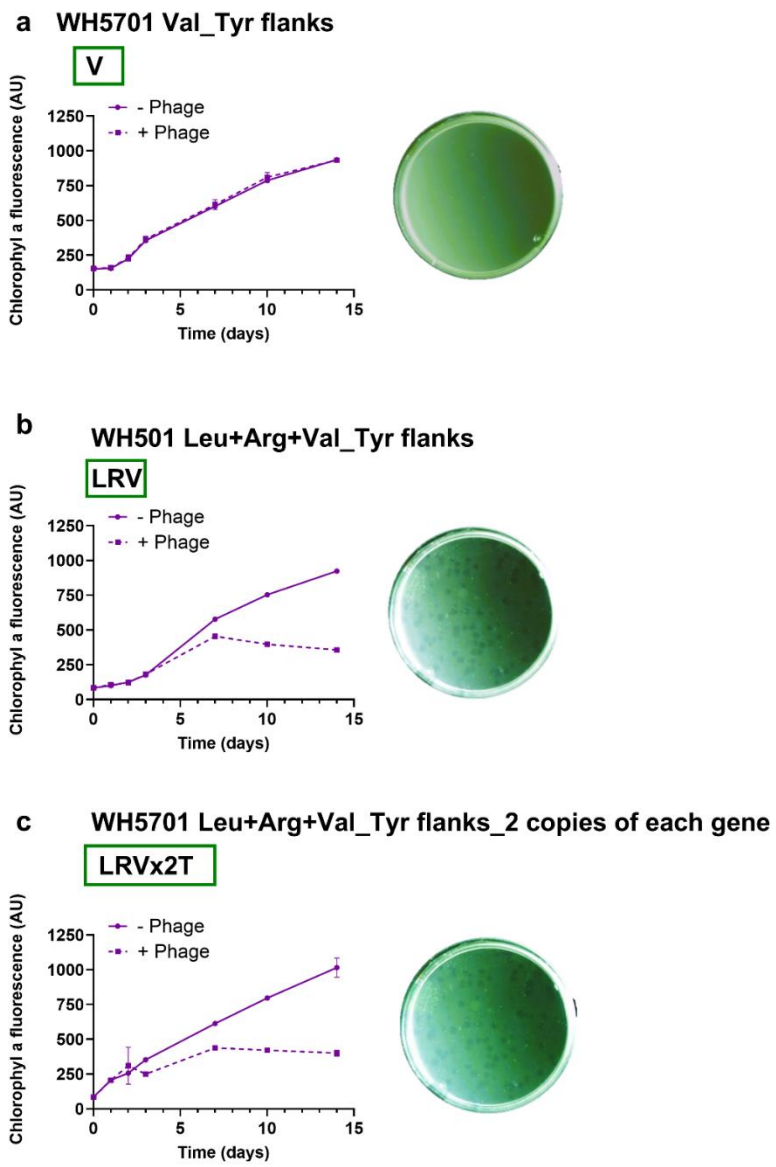
Codons were considered rare if their frequency in the genome is lower than 1% (green) or very rare if their frequency was below 0.5% (blue), following Daniel et al[39].



Extended Data Fig. 1. The effect of the R-M and Gabija defense systems on the sensitivity of *Synechococcus* WH5701 to the S-TIM4 and P-TIM40 phages. Growth of cyanobacteria in the presence S-TIM4 (**a**) and P-TIM40 (**b**). Average and standard deviation of 5 biological replicates. For the R-M system, *hsdR* which encodes the endonuclease was inactivated. For the Gabija system, *gajB* which encodes the helicase was inactivated. AU=arbitrary units.



Extended Data Fig. 2. Codon usage preferences of Syn9 gene expression clusters compared to resistant and sensitive cyanobacteria. The frequency of each codon is the number of times it appears in the genome relative to all codons. Codons were considered rare if their frequency in the genome is lower than 1% (green) or very rare if their frequency was below 0.5% (blue), following Daniel et al.[39]. Codons for which Syn9 encodes tRNA genes are marked with an X. The cyanobacterial strains are marked as sensitive or resistant following Zborowsky and Lindell[17]. Correlation (r) of codon frequencies between Syn9 gene expression clusters and a cyanobacterium is shown. *Syn.*, *Synechococcus; Pro.*, *Prochlorococcus.* These figures show that there is no direct correspondence between resistance and codon usage correlation as there are sensitive *Synechococcus* strains with no correlation to Syn9 gene clusters and *Prochlorococcus* strains that are resistant to Syn9 with codon usage that correlates with Syn9 gene clusters.



Extended Data Fig. 3. **Relevance of tRNA Val^{TAC} for phage sensitivity.** Growth of cyanobacterial strains in the presence and absence of Syn9 (left) and Syn9 plaque formation (right) on *Synechococcus* WH5701 expression strains where the Val^{TAC} tRNA gene (**a**), the Leu^{TAA}, Arg^{TCT} and Val^{TAC} genes in single copy (**b**) or the Leu^{TAA}, Arg^{TCT} and Val^{TAC} genes in two copies (**c**) were cloned with the flanking regions of the WH5701 Tyr^{GTA} tRNA. Growth in liquid is the average and standard deviation of 5 biological replicates. Plates with Syn9 plaques on cyanobacterial lawns are representative images of 3 biological replicates. WH5701 refers to *Synechococcus* WH5701. Boxed color-coded abbreviations match those used in Fig. 3d which shows the schematic representation of the arrangement of tRNA genes and flanking regions in the *Synechococcus* WH5701 plasmid-containing strains.