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

Sophia Zborowsky[1,2] and Debbie Lindell[1*]

[1]Faculty of Biology, Technion – Israel Institute of Technology

[2]Current address: Living Systems Institute, University of Exeter, United Kingdom

* Corresponding author: dlindell@technion.ac.il

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 expression 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 resistance mechanism, 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.

23 **Introduction**

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

33

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

45

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

48 of cyanophages in the oceans[19]. In some regions cyanophages can be a substantial cause of
49 mortality, impacting cyanobacterial distribution, primary production and ocean
50 biogeochemistry[20–23]. In other regions low levels of infection ensue[23,24] and coexistence is
51 considered likely to be due to a high degree of cyanobacterial resistance to co-occurring
52 cyanophages[7,19,25].
53
54 Previously, we reported on a phage – resistant-bacteria interaction where the infection fails
55 post adsorption; the Syn9 cyanophage infecting the cyanobacterium *Synechococcus* sp. strain
56 WH5701 (referred to as *Synechococcus* WH5701 henceforth). Syn9 is a T4-like
57 cyanomyovirus, with a 177.3 Kb genome which encodes for 226 putative proteins and six
58 tRNAs and has a GC content of 40.5%[26]. *Synechococcus* WH5701 is a marine
59 cyanobacterium that belongs to *Synechococcus* subcluster 5.2[27,28]. This cyanobacterium has a
60 2.86 Mb genome that codes for 3,129 proteins with a GC content of 66%[29]. Notably, this
61 cyanobacterium is resistant to all phages tested on it[19,17]. Based on sequence homology it has
62 two known resistance mechanisms, a type I R-M and a Gabija system[5]. However, neither of
63 these systems have been tested for their ability to provide resistance to phage infection in
64 *Synechococcus* WH5701.
65
66 In this study, our primary objective was to unravel the underlying mechanism of intracellular
67 resistance in *Synechococcus* WH5701 against Syn9 infection. We found that the R-M and
68 Gabija systems were ineffective in conferring resistance against this phage. Rather, we found
69 that resistance against Syn9 stemmed from insufficient levels of the Leu^{TAA} tRNA. This was
70 due to dramatically reduced expression of both the cyanobacterial and cyanophage Leu^{TAA}
71 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* WH5701. 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.

Investigating the role of R-M and Gabija systems

We began by examining the two known resistance mechanisms 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]. 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*, and the predicted helicase in the Gabija system, *gajB* (Fig. 1a). If either of these systems were responsible for conferring resistance to the Syn9 phage, gene inactivation would lead to phage sensitivity.

97

98 Upon challenging the *Synechococcus* WH5701 inactivation mutants with Syn9 we found no
99 discernible decline in growth of the cyanobacterium (Fig. 1b). Furthermore, no phage plaques
100 were formed (data not shown). Consequently, we conclude that neither of the two known
101 defense systems is responsible for resistance to the Syn9 phage. These findings support our
102 previous hypothesis that nuclease-based resistance mechanisms are not responsible for
103 *Synechococcus* WH5701 resistance to Syn9 infection.

104

105 *Translational inhibition by tRNA availability*

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

111

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

121

122 Next, we wondered whether *Synechococcus* WH5701 carries the genes for these three tRNAs.
123 We found genes for all three of these tRNAs in the genome of this cyanobacterium. We then
124 asked whether insufficient tRNA availability in *Synechococcus* could be preventing
125 translation of the missing phage proteins. We used reverse transcriptase quantitative PCR
126 (RT-qPCR) to measure tRNAs levels in uninfected *Synechococcus* WH5701 and compared
127 them to those in a strain sensitive to Syn9, *Synechococcus* WH8102. We found that two of the
128 three tRNAs (Leu^{TAA} and Arg^{TCT}) were below the limit of detection. This is more than 4
129 orders of magnitude below tRNA levels for common codons in this cyanobacterium and for
130 these same tRNAs in the sensitive *Synechococcus* WH8102 (Fig. 2a). The third tRNA
131 (Thr^{TGT}), however, was found at high levels in the resistant cyanobacterium despite the
132 corresponding codon being rare in this strain. Interestingly, a fourth tRNA that is relatively
133 rare in *Synechococcus* WH5701 (Val^{TAC}) was also below detection limits, even though its
134 codon is not differentially distributed in present and absent Syn9 phage proteins. Thus, despite
135 the presence of the relevant tRNA genes in the genome of *Synechococcus* WH5701, the tRNA
136 levels of some of them were undetectable.
137
138 Phages often carry tRNA genes, including Syn9, which is thought to compensate for the
139 mismatch between their codon usage and the tRNA pool of the host[26,30,31]. Interestingly, the
140 four tRNAs discussed above are among the six coded in the Syn9 genome. Therefore, we
141 wondered whether these phage tRNAs were expressed in the resistant cyanobacterium. We
142 found detectable levels of all six phage tRNAs during infection, but these were approximately
143 1000-fold lower in the resistant *Synechococcus* WH5701 relative to the sensitive
144 *Synechococcus* WH8102 (Fig. 2b). These findings suggest that the phage tRNAs were not
145 sufficiently transcribed or did not accumulate in the resistant cyanobacterium, and would not
146 compensate for the low levels resulting from the cyanobacterial genome.

147

148 Given the overall low levels of these tRNAs, we hypothesized that *Synechococcus* WH5701 is
149 resistant to Syn9 because the tRNAs required to translate key phage proteins are not present at
150 sufficient levels. This could be due to insufficient tRNA transcription or rapid degradation
151 after transcription. If there is no, or low, transcription then expressing the tRNAs using
152 appropriate regulatory elements would result in tRNA accumulation in the cells. Conversely,
153 if the tRNAs are targeted for degradation post transcription, then additional regulatory
154 elements for transcription would not result in an accumulation of the tRNAs.

155

156 To test this, we expressed two undetectable *Synechococcus* WH5701 tRNA genes, Leu^{TAA} and
157 Arg^{TCT}, together from a replicative plasmid under the control of the cyanobacterial *rnpB*
158 promoter. Our insert included the sequence coding for the tRNA itself as well as the flanking
159 regions since these can influence stability through post-transcriptional regulation. We found
160 that the two tRNAs expressed from the vector were present at levels that were at least 4-
161 orders of magnitude above those in the resistant wild-type (WT) *Synechoccous* WH5701.
162 However, tRNA levels were still well below those in the sensitive *Synechococcus* WH8102
163 (approximately 10-fold lower for Leu^{TAA} and two-fold lower for Arg^{TCT}) (Fig. 3a, b). These
164 findings indicate that the tRNAs were not targeted for degradation post-transcriptionally in
165 this expression strain and suggests that they are likely not transcribed in the WT resistant
166 strain.

167

168 To test if the presence of these tRNAs was sufficient to allow infection, we exposed the
169 expression strain to the Syn9 cyanophage. We observed a clear decline in growth of the
170 expression strain, similar to that for the sensitive *Synechococcus* WH8102 WT and different
171 to the resistant *Synechococcus* WH5701 WT (Fig. 4). Furthermore, plaques were formed on

172 lawns of the expression strain (Fig. 4c). However, the number of phages produced was
173 considerably fewer than in the sensitive strain as seen from the efficiency of plating (EOP) of
174 1.2% relative to 100% in the sensitive strain (p value<0.0001) (Fig. 3c). This lower efficiency
175 of plating could be due to the lower tRNA levels in this expression strain. Irrespective of the
176 number of phages produced, these findings unequivocally show that expression of these two
177 tRNAs resulted in phage sensitivity and that *Synechococcus* WH5701 resistance to Syn9 is
178 due, at least partially, to insufficient levels of specific tRNAs. Our findings further suggest
179 that neither specific protein degradation nor specific tRNA degradation are the mechanism of
180 resistance.
181
182 *Investigating transcriptional loss of tRNAs in* Synechococcus *WH5701*
183 We set out to understand what might be causing the lack of expression of the cyanobacterial
184 tRNA genes. We hypothesized that these genes have lost necessary regulatory motifs for their
185 expression. To assess this possibility, we started by cloning the two tRNA genes, Leu^{TAA} and
186 Arg^{TCT}, with new flanking regions, replacing these regions with the flanking regions from a
187 tRNA gene, Tyr^{GTA}, which is highly expressed in *Synechococcus* WH5701 (see Fig. 2a). The
188 insert was not cloned with an external promoter to assess whether the flanking regions
189 themselves contained the necessary information for transcription. The levels of both tRNAs
190 were present in high amounts, considerably higher than when these same tRNA genes were
191 under the control of the *rnpB* promoter (Fig. 3a, b). However, they were still somewhat lower
192 than those found in *Synechococcus* WH8102, 4-fold for Leu^{TAA} and 1.25-fold for Arg^{TCT}.
193 Thus, the Tyr^{GTA} flanking regions were sufficient for transcription and resulted in relatively
194 high levels of tRNAs.
195

196 Next, we looked for motifs upstream of tRNAs that could be responsible for expression. This
197 was done for the seven tRNAs for which transcription was experimentally verified by RT-
198 qPCR (Fig. 2a). We found a conserved motif, TXXG, in the -35 position of all seven
199 transcribed tRNAs. This motif was absent, however, from the upstream region (from the
200 transcription start site to the -35 position) of the three undetected tRNAs, Leu^{TAA}, Arg^{TCT}, and
201 Val^{TAC} (Fig. 2a). To test if this motif was needed for transcription, we replaced it with AAAA
202 nucleotides in the upstream region of the inserts with the Tyr^{GTA} flanking regions. Indeed,
203 transcription was lost when the TXXG was removed (Fig. 3a, b). However, transcription did
204 not result when we added this motif to the native Leu^{TAA} and Arg^{TCT} tRNA genes in the -35
205 position (Fig. 3a, b). These findings indicate that the TXXG motif is essential but not
206 sufficient for transcription, and that other regulatory elements in the tRNA flanking regions
207 are also necessary for tRNA transcription.
208
209 We then assessed whether tRNA expression with and without this -35 motif affected
210 sensitivity to Syn9. We challenged the various *Synechococcus* WH5701 expression strains
211 with Syn9 and found that expression of the tRNAs with the Tyr^{GTA} flanking regions resulted
212 in sensitivity to Syn9, with both a decline in growth of the cyanobacterium and the production
213 of plaques (Fig. 5a). For the expression strains in which tRNA transcription did not result, no
214 sensitivity to the phage was found, neither through a decline in cyanobacterial growth nor
215 through plaque formation (Fig. 5b, c). These findings further indicate that tRNA expression is
216 sufficient to render *Synechococcus* WH5701 sensitive to Syn9 infection.
217
218 As described above, all *Synechococcus* WH5701 expression strains that resulted in tRNA
219 transcription were also sensitive to Syn9. However, there was a pronounced difference in the
220 number of phages produced. Expression of the tRNAs with the Tyr^{GTA} flanking regions

221 produced almost 30-fold more phages than when expressed from the *rnpB* promoter, with an
222 efficiency of plating of 35% versus 1.2% respectively (p value<0.0001) (Fig. 3c). Since there
223 was a gradient both in tRNA levels and phage production, we wondered whether phage
224 production is directly related to tRNA transcript levels. To assess this, we cloned two copies
225 of the two tRNA genes (Leu^{TAA} and Arg^{TCT}), each set with its own Tyr^{GTA} flanking regions
226 into *Synechococcus* WH5701. We observed about a 2-fold increase in transcript levels (Fig.
227 3a, b), but there was no effect on phage production (Fig. 3c, p=0.7 for U-test for EOP of
228 expression strains with single and double gene copies). Thus, tRNA transcript levels do not
229 correlate directly to phage production, suggesting that other factors also affect phage
230 production.
231
232 Finally, we were interested in determining whether both tRNAs were required for the
233 sensitive phenotype. We expressed each tRNA separately with the Tyr^{GTA} flanking regions
234 and saw that expression of Leu^{TAA} alone was sufficient to render *Synechococcus* WH5701
235 sensitive to Syn9 (Fig. 5d). In contrast, expression of Arg^{TCT} alone left the cyanobacterium
236 resistant to the Syn9 phage (Fig. 5e). However, phage production was approximately double
237 when both tRNAs were expressed than when Leu^{TAA} was expressed alone, with an efficiency
238 of plating of 18% with Leu^{TAA} alone versus 35% with both tRNAs (p=0.05, Fig. 3c). Hence
239 while expression of Arg^{TCT} alone did not result in phage sensitivity, this tRNA contributed
240 significantly to phage production.
241
242 **Discussion**
243 Our findings indicate that the lack of expression of the Leu^{TAA} gene in *Synechococcus*
244 WH5701 confers it with resistance to infection by Syn9. This lack of expression resulted in
245 the inability of the phage to translate all of its essential proteins[17] and was the result of the

246 lack of an essential promoter motif in the flanking regions of the tRNA gene (this study). This
247 can be considered a passive mechanism of resistance since it is caused by the loss of
248 expression of a component in the cell that is required by the phage and also does not affect the
249 growth of the cell[17]. This mechanism of resistance acts at a late stage of infection, since Syn9
250 gets as far as building capsids[17] . Thus, this passive mechanism of resistance is not the result
251 of early stage incompatibility between this cyanophage and cyanobacterium.
252
253 The most well-known passive resistance mechanisms in bacteria arise from changes in an
254 outer cellular component that phages use as receptors, thus preventing phage adsorption and
255 entry into the cell[6,7–11], and thus function extracellularly. Intracellular mutations or loss-of-
256 function of cellular components as a means of resistance is rarely reported in bacteria, but
257 based on our findings, may be more common than currently appreciated. For example, we
258 previously reported that many marine cyanophages enter into marine cyanobacteria but cannot
259 complete their infection cycle[17]. Most of these cyanobacteria (12 out of 16 marine
260 *Synechococcus* and *Prochlorococcus* strains) lack recognizable active mechanisms of
261 resistance[17]. At this stage, it remains unknown whether the means of resistance in these cells
262 are passive intracellular mechanisms, similar to that reported here, or whether they carry
263 currently unknown active resistance systems. While recent research has uncovered a plethora
264 of new systems of active resistance in bacteria[5,6,32], little is known about potential passive
265 intracellular mechanisms for bacteria-at-large. Thus, further investigation into this
266 phenomenon has the potential to significantly enhance our understanding of phage-bacteria
267 interactions and the evolutionary dynamics underlying bacterial resistance to phages.
268
269 Defense mechanisms that target translation have been demonstrated previously against
270 phages. These are active mechanisms that, for example, cleave essential host translation

271 factors[33] or target tRNAs for degradation[34]. Furthermore, these are abortive infection systems
272 that result in the death of the infected cell and provide protection to the population by
273 preventing the spread of the phage[35]. This is in stark contrast to the means of resistance
274 reported here which is both passive and does not result in the death of the cyanobacterium.
275 Thus, it is now clear that both active and passive, abortive and non-abortive, defense
276 mechanisms work at the level of translation inhibition.
277
278 In this study we focused our work on investigation of the expression of the cyanobacterial
279 tRNAs. While restoring expression of the leucine and arginine tRNAs was sufficient to result
280 in phage sensitivity in *Synechococcus* WH5701, phage production was still considerably
281 lower than in the naturally sensitive *Synechococcus* WH8102 strain. This may, in part, be due
282 to the orders of magnitude lower expression of all six of the phage tRNAs, including the
283 phage leucine and arginine tRNAs (Fig. 2b). It could also be due to low transcription of many
284 Syn9 transcript in this resistant strain, ~10-110% of that in the sensitive strain[17]. This
285 disparity in tRNA and mRNA transcript levels could stem from low transcriptional
286 compatibility of Syn9 to *Synechococcus* WH5701. However, it is also possible that this lower
287 efficacy of transcription is the result of a currently unknown active mechanism of resistance.
288 It is feasible that the two known active defense systems in the genome of *Synechococcus*
289 WH5701 confer resistance against other phages, as may the lack of transcription of the Val^{TAC}
290 tRNA (Fig. 2a). Indeed, no currently known phage can successfully infect *Synechococcus*
291 WH5701[17,19]. It appears that this *Synechococcus* strain has evolved to possess multiple layers
292 of defense that protect it against a wealth of cyanophages, culminating in the emergence of a
293 cyanobacterium that is a phage-resistant specialist.
294

295 The evolution of the lack of expression of certain tRNA genes in *Synechococcus* WH5701 is
296 likely to have occurred gradually. Clearly, a phage's ability to translate its proteins and
297 successfully infect a host is dependent on its ability to utilize the tRNA pool of the host[36,37].
298 Since lytic phages kill off the cell, it is considered likely that phages exert selective pressure
299 on the host that influences its codon usage pattern and leads to differentiation from that of the
300 phage[38,37,31]. Our study provides experimental evidence in support of this hypothesis.
301
302 Combining our data with theoretical evidence from the literature, we propose a multi-step
303 scenario that can explain the evolutionary process that led to the loss of tRNA expression as
304 an effective means of cyanophage resistance in *Synechococcus* WH5701 and perhaps other
305 bacteria. Phages and other parasites often have low GC content which is potentially due to the
306 lower cost of producing AT rich genomes and the higher availability of these nucleotides[39].
307 We hypothesize that the cyanobacterium underwent adaptations that modify its codon usage
308 patterns to differ from those most critical for the expression of phage replication and
309 morphogenesis genes[30,36,31]. This is likely to have preceded, or occurred simultaneously with
310 the downregulation of tRNA gene expression[40–43] through the loss of at least the TXXG motif
311 at the -35 position of three key tRNA genes (Fig. 2a). These tRNAs are among those that
312 match the most common codons in T4-like cyanophage genomes[31] and thus their lack of
313 expression is likely to have conferred resistance against infection by T4-like cyanophages,
314 such as Syn9. This deficiency would exert substantial selective pressure for the acquisition
315 and expression of the relevant tRNA genes by cyanophages, to compensate for such codon
316 usage discrepancies[30,31] and allow for infection. Subsequently, we propose that
317 *Synechococcus* WH5701 developed a presently unknown mechanism to limit expression of
318 cyanophage tRNA genes, such that their presence was rendered ineffectual, preventing
319 infection. Thus, in this scenario, the evolution of resistance in this cyanobacterium stemmed

320 from the gradual loss of tRNA gene expression, whether encoded by the cell or acquired by
321 the phage, underscoring the dynamic interplay between bacterial hosts and phages in shaping
322 their co-evolutionary trajectories.
323
324 **Methods**
325 *Cyanobacterial growth and cyanophage propagation*
326 *Synechococcus* WH5701 and WH8102 cultures were grown in an artificial seawater medium
327 (ASW)[17]. Cultures were grown at 21°C at a light intensity of 15-25 µmol photons·m^{-2}·s^{-1}
328 under a 14:10 light:dark regime. Culture growth was measured using chlorophyll *a*
329 autofluorescence (excitation/emission: 440/680 nm) as a proxy for biomass using a Synergy 2
330 microplate reader (BioTek).
331
332 For isolation of conjugated colonies, pour plating was used, the cyanobacterial culture was
333 serially diluted and mixed with ultra-pure low-melting point agarose (Invitrogen) at 0.28% in
334 growth medium which was also supplemented with 1 mM sodium sulfite. The helper
335 bacterium, *Alteromonas sp.* strain EZ55, was added to the plates to obtain high plating
336 efficiency[44].
337
338 The Syn9 cyanophage was propagated by infecting a sensitive strain at a low multiplicity of
339 infection (MOI) of <0.01 and allowing the culture to clear. Cells were removed from the
340 phage lysates by filtration through a 0.2 µm pore-sized filter. For small volumes (< 20 ml)
341 Acrodisc syringe filters (PALL) were used; for large volumes Nalgene Rapid Flow 50 mm
342 filter units (Thermo Scientific) were used.
343

344 The titer of the lysate (the concentration of infective phages) was determined by plaque assay.
345 The phage lysate was serially diluted and mixed with the sensitive *Synechococcus* strain
346 WH8102 at a concentration of 2×10^6 in pour plates (as described above, but without the
347 addition of the EZ55 helper bacterium) to form a cyanobacterial lawn on which clearings in
348 the lawn (plaques) could be counted. The plates were incubated at cyanobacterial growth
349 conditions until plaque clearings were observed in the lawn. Plaques were counted until no
350 new plaques appeared.
351
352 *Insertional inactivation of* Synechococcus *WH5701 genes*
353 The Type I restriction system was inactivated by disrupting the restriction subunit gene (*hdsR*;
354 protein ID WP_071934184.1), while the Gabija system was inactivated by interrupting the
355 helicase gene (*gajB*; protein ID WP_071934305.1). Gene inactivation was achieved through
356 plasmid integration into the host chromosome via a single homologous recombination event
357 following Brahamsha[45]. A 250 bp homologous segment was modified to include stop codons
358 in all three reading frames to ensure gene inactivation (see Fig. 1a) and was cloned into a
359 pBR322-derived plasmid[45] (named pDS1)[46]. The chloramphenicol resistance gene in this
360 plasmid was codon optimized for expression in cyanobacteria[47]. The plasmid was
361 electroporated into the S17 *E. coli* donor strain[48] and colonies were selected on LB plates with
362 100 μg/ml ampicillin. The plasmid was then mobilized into *Synechococcus* WH5701 by
363 conjugation. Colony formation was performed using pour-plating in the presence of 2 μg/ml
364 chloramphenicol. Successful gene interruption was confirmed by PCR using primers within
365 the plasmid and upstream or downstream of the crossover region in the cyanobacterium (see
366 Table S2 for primer sequences). To ensure full segregation (disruption of all copies of the
367 cyanobacterial chromosome) PCR was performed to verify the absence of wild-type gene
368 copies. Disruption of transcription was verified by RT-qPCR using primers flanking the site

369 of recombination (see Table S2 for primer sequences). Further details on RT and qPCR are
370 provided below.
371
372 *Analyzing host and phage codon usage and motif searches*
373 Codon usage in the genomes of *Synechococcus* WH5701, *Synechococcus* WH8102, and Syn9
374 was analyzed with the frequency of each of the 61 codons counted. Additionally, for phages,
375 codon usage per gene was assessed, comparing the average occurrence of codons between
376 translated and untranslated phage proteins in *Synechococcus* WH5701, as per Zborowsky and
377 Lindell[17]. The determination of codon usage was carried out using the Countcodon program
378 version 4 with default parameters for bacteria
379 (https://www.kazusa.or.jp/codon/countcodon.html). Statistical comparisons of codon usage
380 per gene were conducted using the Mann-Whitney U test.
381
382 The TXXG motif was identified after manual inspection of the upstream region of the 10
383 tRNA genes in *Synechococcus* WH5701 for which tRNA levels were investigated using RT-
384 qPCR. The upstream regions of the 7 expressed tRNAs were compared to the 3 tRNA genes
385 that were not expressed in this cyanobacterium.
386
387 *Expression of tRNA genes in* Synechococcus *WH5701*
388 To explore the impact of the absence of Leu^{TAA} and Arg^{TCT} tRNAs on resistance in
389 *Synechococcus* WH5701, these genes were expressed from a replicative plasmid with
390 different regulatory elements. (1) Native tRNA sequences and flanking regions of the Leu^{TAA}
391 and Arg^{TCT} genes as present in the *Synechococcus* WH5701 genome, with the addition of the
392 *rnpB* promoter upstream of the first tRNA gene (WH5702 *rnpB* promoter). (2) The same
393 tRNA sequences as in (1) but without the *rnpB* promoter and with flanking regions of each

394 gene replaced with those of the Tyr^{GTA} tRNA from *Synechococcus* WH5701, which include
395 the TXXG motif in the -35 position relative to the transcription start site (WH5701 Tyr flanks
396 and WH5701 Tyr flanks two copies). (3) The same as in (2) but with the TXXG motif
397 replaced with AAAA (WH5701 Tyr flanks TXXG replaced with AAAA). (4) Native tRNA
398 sequences and flanking regions but with the addition of TXXG to the -35 position (WH5701
399 native flanks TXXG added to -35). (5) The same as in (2) but for only one of the tRNA genes
400 (WH5701 Leu alone Tyr flanks or WH5701 Arg alone Tyr flanks). Constructs were
401 synthesized and cloned into a pUC57-Amp plasmid (Genewiz) and were then inserted into the
402 pDS-ProCAT plasmid[47] using restriction enzymes. The pDS-ProCAT plasmids were then
403 electroporated into *E. coli* S17 and subsequently conjugated into *Synechococcus* WH5701 as
404 described above. Selection of positive colonies were done as described above, except that *E.*
405 *coli* S17 colonies harboring the plasmid were selected on LB plates supplemented with 750
406 µg/ml erythromycin (Em).
407
408 *Determining transcription levels by reverse transcription*
409 For assessing tRNA levels and the expression of interrupted R-M and Gabija genes, total
410 RNA was extracted from cyanobacterial cells. Samples were taken 5 hours after phage
411 infection, as were non-infected controls, and were centrifuged at 9,000 g for 15 minutes at
412 4ºC. Cells were immediately flash-frozen in liquid nitrogen and stored at -80°C until RNA
413 extraction.
414
415 For RNA extraction, cells were thawed on ice and resuspended in a solution containing 10
416 mM Tris-HCl pH 8, 100 units of RNase inhibitor (Applied Biosystems), and 15,000 units of
417 Lysozyme (Sigma). After incubating at 37°C for 30 minutes for initial cell wall degradation,
418 RNA was isolated using the Quick RNA Mini Prep kit (Zymo). To remove residual DNA, the

419 Turbo DNA Free kit (Invitrogen) was used, incubating with DNase at 37°C for 1 hour.
420 Reverse transcription was conducted with random hexamer primers using the High Capacity
421 cDNA Reverse Transcription kit (Applied Biosystems). The RT procedure included primer
422 extension at 25°C for 10 minutes, cDNA synthesis at 37°C for 120 minutes, and reaction
423 termination at 85°C for 5 minutes. Samples were diluted five-fold in 10 mM Tris-HCl pH 8
424 and stored at -20°C before real-time quantitative PCR. The *Synechococcus* WH5701 *rnpB*
425 gene served as a positive control for reverse transcription and as a standardizing marker for
426 gene expression. No RT controls were performed on all samples to ensure that reported
427 transcript levels did not originate from residual phage DNA.
428
429 *Real-time quantitative PCR*
430 PCR was conducted using the LightCycler 480 Real-Time PCR System (Roche). The cycling
431 program began with a denaturation step of 95°C for 10 minutes, followed by 35-40 cycles of
432 amplification. Each cycle involved denaturation at 95°C for 10 seconds, annealing at 52°C-
433 68°C for 10 seconds, and elongation at 72°C for 10 seconds. Fluorescence was measured for
434 each reaction (FAM; Ex/Em 465/510nm) at the end of each cycle. The LightCycler 480
435 software (release 1.5.0) was used to calculate the point at which the fluorescence of a sample
436 exceeded the background fluorescence (Cp) using the absolute quantification/2nd-derivative
437 maximum analysis package. Melting curve analysis on the LightCycler 480 instrument
438 verified the specificity of the amplified PCR product.
439
440 Each qPCR reaction contained 1X LightCycler 480 SYBR Green I Master mix (Roche), 200
441 nM desalted primers, and 5 µl template (in 10 mM Tris-HCl pH8) in a total reaction volume
442 of 20 µl (see Table S2 for primer sequences). The number of DNA copies in the reaction was
443 determined by comparing the Cp to that of a sample with a known DNA concentration using a

444 standard curve. Genomic DNA for standard curves was extracted using the DNeasy Blood &
445 Tissue kit (Qiagen) from cyanobacterial strains and a phenol:chloroform based method for the
446 phages. DNA concentrations in ng/ml were measured by absorbance at 260 nm using a
447 Synergy 2 microplate reader (BioTek) and converted to gene copies/ml by inputting the
448 genome length of the cyanobacterium or phage into the URI Genomics & Sequencing Center
449 calculator for determining the number of copies.
450
451 *Determining resistance or sensitivity to phage infection*
452 To assess sensitivity or resistance to each phage, cultures were exposed to phage in liquid
453 medium, and their growth was monitored for 2 weeks. To maintain consistent phage-bacteria
454 ratios at the beginning of the experiments, cells were quantified using flow cytometry with the
455 Influx flow cytometer (Becton Dickinson), based on forward scatter and autofluorescence
456 (emission at 692/640nm). Yellow-green 1 μm diameter microspheres (Fluoresbrite) served as
457 internal standards for size and fluorescence consistency. Culture growth was monitored using
458 chlorophyll *a* fluorescence (as described above). Furthermore, sensitivity to the phage was
459 tested by plaque assay. A strain was considered sensitive if there was either reduced growth in
460 liquid relative to uninfected controls or visible clearings in plaque assays or deemed resistant
461 if there was no growth reduction in liquid medium and no plaque formation on cyanobacterial
462 lawns.
463
464 *Statistical analysis*
465 All statistical analyses were performed using the IBM SPSS statistics version 24. Mann-
466 Whitney's U test (nonparametric) was used. Normality was tested using the Shapiro Wilk test
467 for normality. Equality of variance was tested using the Levene's test using SPSS software.
468

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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 analyzed the data. S.Z. and D.L. wrote the manuscript.

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599

600 **Table 1. Codon usage and frequency for select tRNAs in *Synechococcus* and Syn9 genomes**
601

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	0.10	0.34	2.13	1.4	3.8	0.0006
AGA	Arg	TCT	TCT	0.73	1.14	2.47	2.6	4.9	0.012
ACA	Thr	TGT	TGT	0.15	1.12	1.82	3.3	4.9	0.049
GUA	Val	TAC	TAC	0.31	0.46	1.43	2.4	4.1	0.59
GCA	Ala	TGC	TGC	1.75	2.31	1.87	4.7	8.2	0.31
AAC	Gln	GTT	GTT	0.70	1.59	2.36	3.6	6.2	0.34
UAC	Tyr	GTA		2.30	0.48	1.43	1.9	3.2	0.55
GGC	Gly	GCC		3.50	2.91	0.78	0.9	4.2	0.14
CGG	Arg	CCG		2.48	2.31	0.48	0.2	0.3	0.106
GCC	Ala	GGC		3.47	2.94	0.34	1.0	1.3	0.19

602 [a]The ten tRNAs who levels were measured in Fig. 2a are shown here. See Table S1 for a complete list
603 of codons and their frequencies.
604 [b]The frequency of each codon is the number of times it appears in the genome relative to all codons.
605 [c]The number of times a codon appears per gene in Syn9 proteins that were translated (n=81) versus
606 proteins that were not translated (n=35) during infection of *Synechococcus* WH5701 was compared
607 using the Mann Whitney U test.

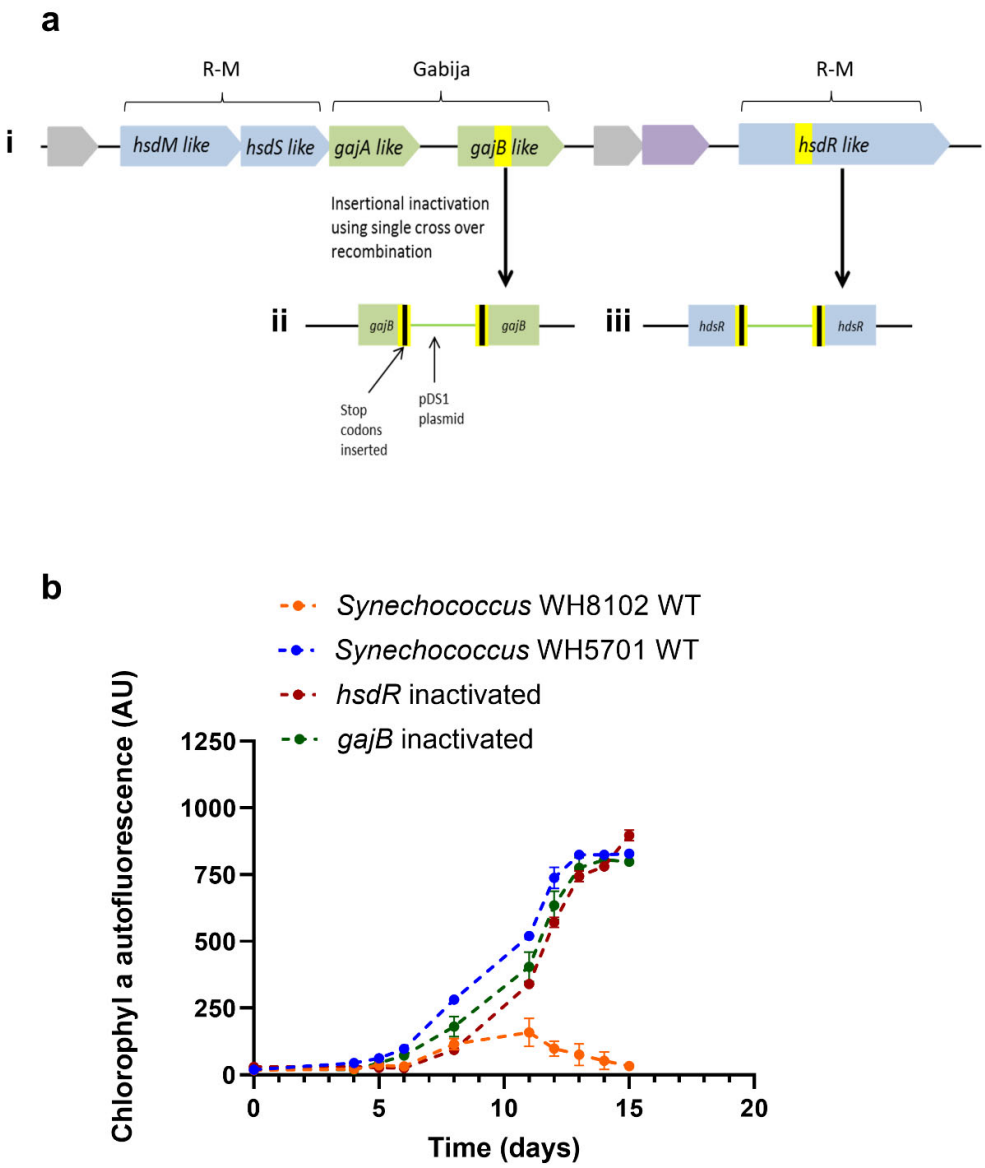

Figure 1. The effect of known defense system genes on the sensitivity of *Synechococcus* WH5701 to the Syn9 phage. **a** Schematic representation of the organization of the two defense systems in the *Synechococcus* WH5701 genome (i) and in the relevant region in the Gabija system (ii) and R-M system (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.

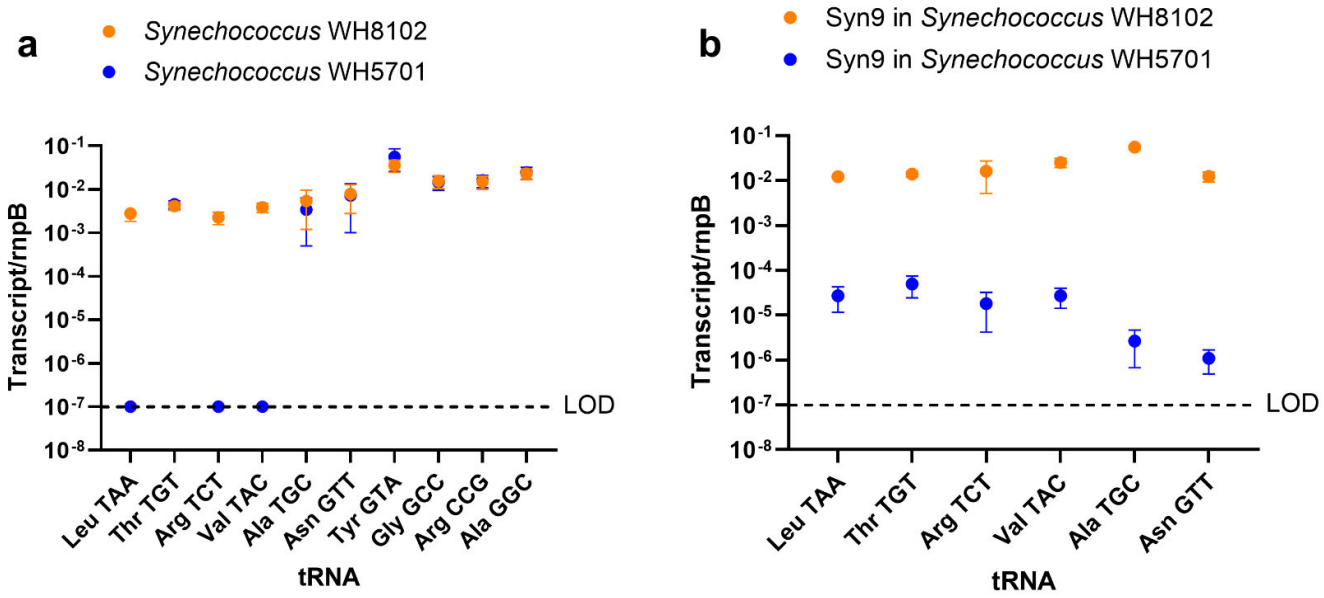

Figure 2. **Phage and cyanobacterial tRNA levels. a.** Levels of cyanobacteria-encoded tRNAs during growth (without infection) for the 6 tRNA genes which have homologues in the Syn9 phage (Leu^{TAA}, Thr^{TGT}, Arg^{TCT}, Val^{TAC}, Ala^{TGC}, Asn^{GTT}) and 4 tRNA genes with common codons in the cyanobacterial 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. Average and standard deviation of 3 biological replicates. LOD - limit of detection of the RT-qPCR is approximately 100 transcripts. All data is normalized to levels of cellular *rnpB.*

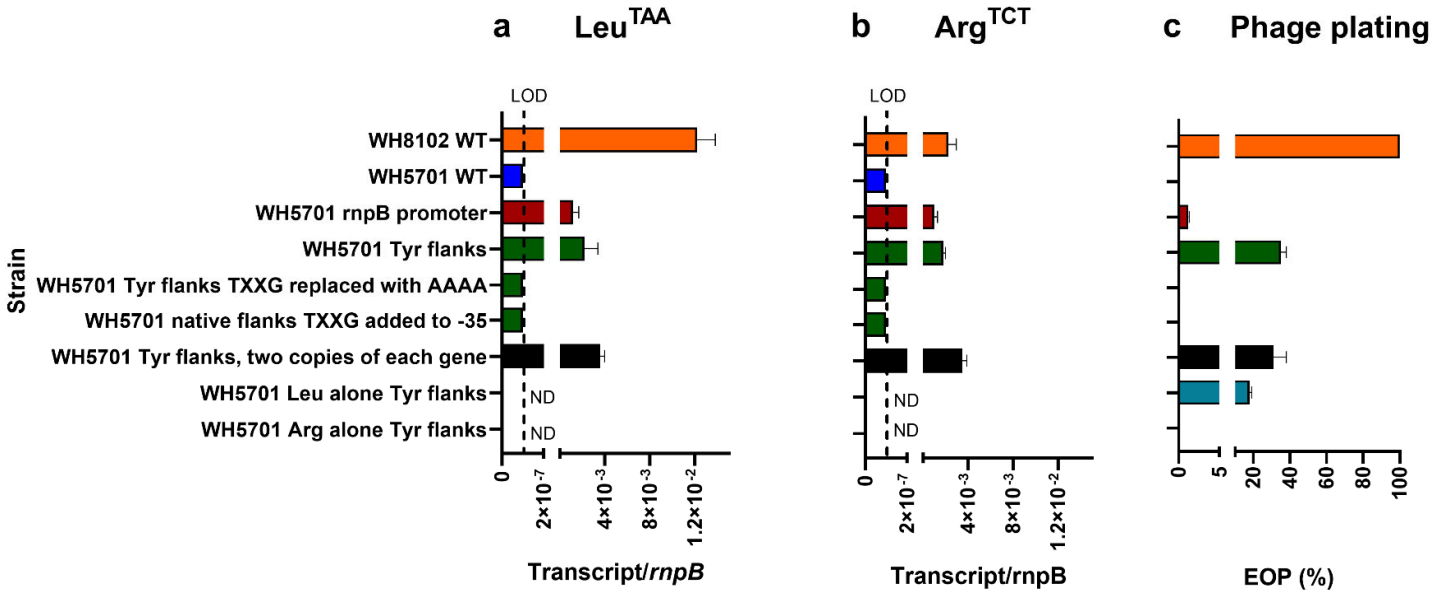

Figure 3. Expression of Leu^{TAA} and Arg^{TCT} tRNAs from, and efficiency of plating on, expression strains. Transcript levels of the Leu^{TAA} tRNA (**a**) and the Arg^{TCT}gene (**b**), and efficiency of plating (EOP) of Syn9 on expression strains relative to the sensitive *Synechococcus* WH8102 (**c**). Average and STD of 3 biological replicates. Colors match those used in Figures 4 and 5, where relevant. LOD, limit of detection (10^{-7} transcripts/*rnpB*). ND, not determined. WH8102 and WH5701 refer to *Synechococcus* WH8102 and *Synechococcus* WH5701, respectively.

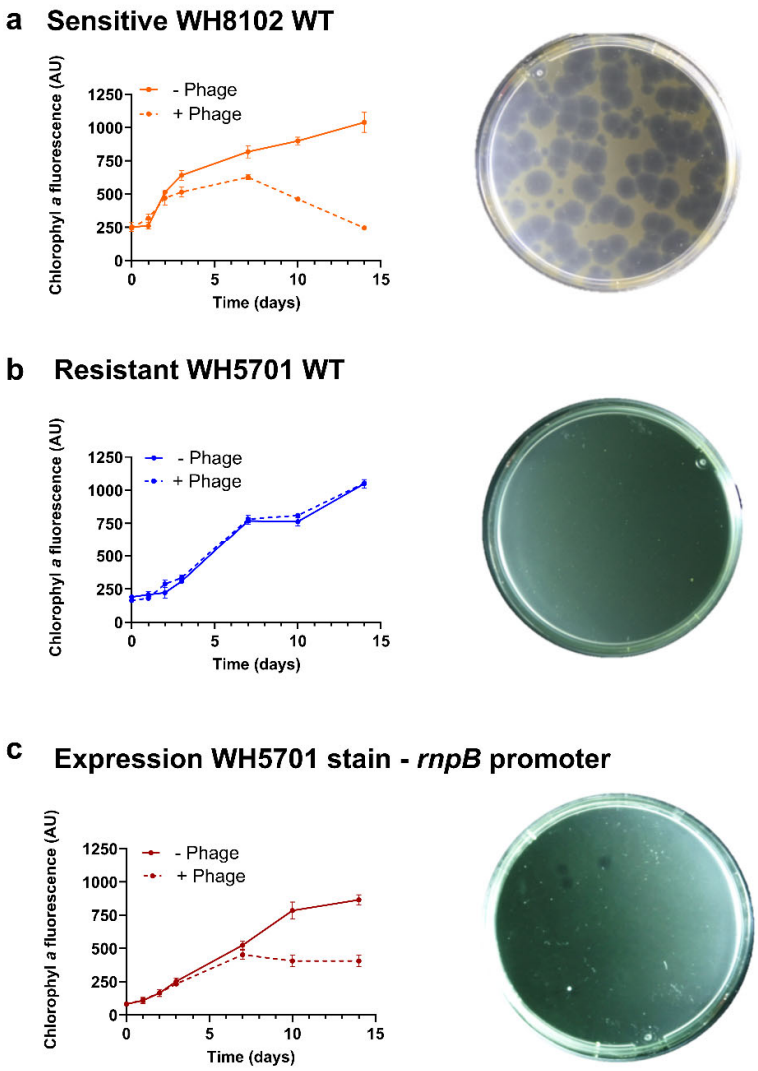

Figure 4. **Sensitivity of *Synechococcus* WH5701 tRNA expression strain with *rnpB* promoter to infection by Syn9.** Growth of cyanobacterial strains in the presence and absence of Syn9 (left) and plaque formation of Syn9 (right) on sensitive *Synechococcus* WH8102 WT (**a**)**,** resistant *Synechococcus* WH5701 WT (**b**), and WH5701 expression strain where the tRNA genes were cloned under a *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 *Synechococcus* WH8102 and *Synechococcus* WH5701, respectively.

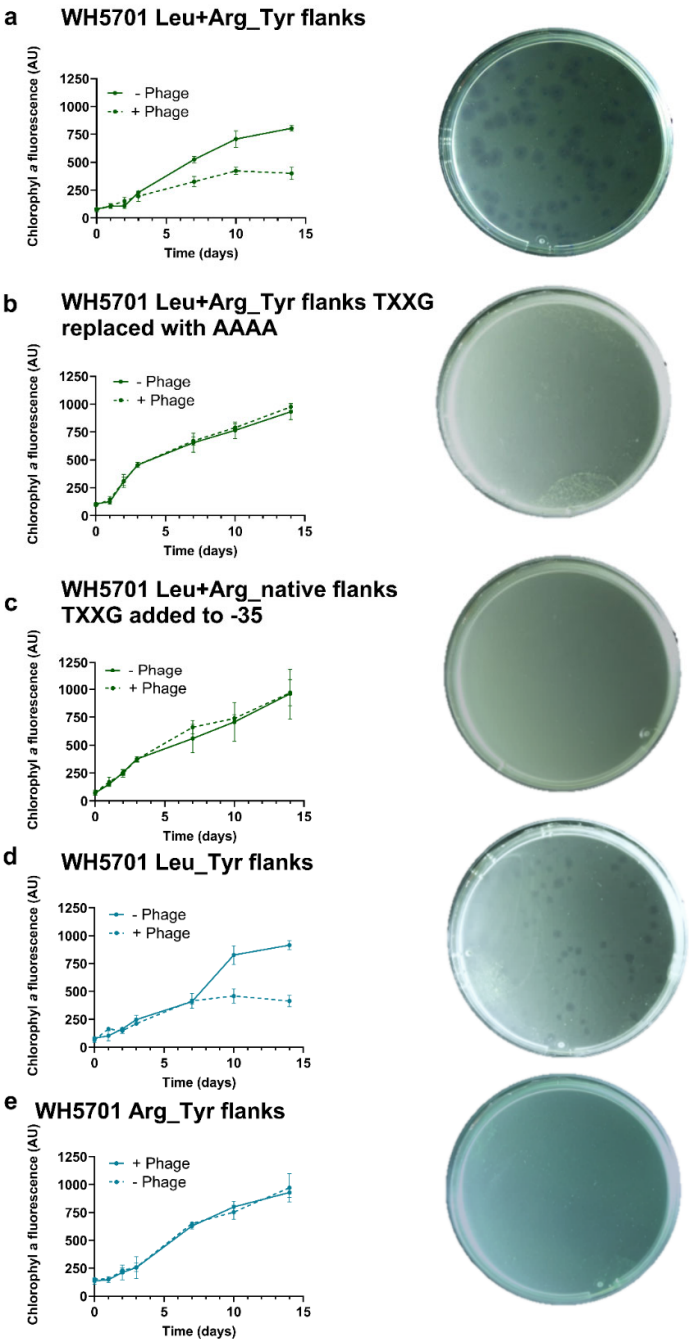

Figure 5. **Relevance of tRNA flanking regions for tRNA transcription and phage sensitivity.** Growth of cyanobacterial strains in the presence and absence of Syn9 (left) and Syn9 plaque formation (right) on *Synechococcus* WH5701 expression strains where the Leu and Arg tRNA genes were cloned: with the WH5701 Tyr^{GTA} tRNA flanking regions (that naturally contain the TXXG motif at the -35 position relative to the TSS) (**a**), with the Tyr^{GTA} tRNA flanking regions where the TXXG motif was replaced with AAAA (**b**), and with the native Leu and Arg flanking regions where the TXXG motif was inserted at the -35 position (**c**). *Synechococcus* WH5701 expression strain with the Leu tRNA gene alone with Tyr flanking regions (**d**). *Synechococcus* WH5701 expression strain with the Arg tRNA gene alone with Tyr flanking regions (**e**). 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.