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A swapped genetic code prevents viral infections and gene transfer

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Summary

Engineering the genetic code of an organism has been proposed to provide a firewall from natural ecosystems by preventing viral infections and gene transfer^{1–6}. However, numerous viruses and mobile genetic elements encode parts of the translational apparatus^{7–9}, potentially rendering a genetic-code-based firewall ineffective. In this paper, we show that such mobile transfer RNAs (tRNAs) enable gene transfer and allow viral replication in *Escherichia coli* despite the genome-wide removal of three of the 64 codons and the previously essential cognate tRNA and release factor genes. We then establish a genetic firewall by discovering viral tRNAs that provide

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Author contribution

A.N. developed the project, led analyses, and wrote the manuscript with input from all authors. A.N. and G.M.C supervised research. S.V. performed tRNA-Leu suppressor screens, sfGFP expression assays, assisted in the construction of the pLS plasmids and biocontainment experiments. R.F. assisted in experiments, performed adaptive laboratory evolution and growth rate measurements. S.V.O., E.R., and M.B. provided environmental samples for phage isolation, performed replication assays, and provided support for phage experiments and genome analyses. M.L., K.C., F.H. performed DNA synthesis, while M.B.T., A.C.P., and E.K. supported the project. B.B. performed tandem mass spectrometry analyses. K.N. and J.A.M. provided reagents for tRNAseq experiments.

Competing Interests statement

The authors declare competing financial interests. Harvard Medical School has filed a provisional patent application related to this work on which A.N., S.V., and G.M.C. are listed as inventors. M.L., K.C., and F.H. are employed by GenScript USA Inc., but the company had no role in designing or executing experiments. G.M.C. is a founder of the following companies in which he has related financial interests: GRO Biosciences, EnEvolv (Ginkgo Bioworks), and 64x Bio. Other potentially relevant financial interests of G.M.C. are listed at <http://arep.med.harvard.edu/gmc/tech.html>.

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exceptionally efficient codon reassignment allowing us to develop cells bearing an amino-acid-swapped genetic code that reassigns two of the six serine codons to leucine during translation. This amino-acid-swapped genetic code renders cells resistant to viral infections by mistranslating viral proteomes and prevents the escape of synthetic genetic information by engineered reliance on serine codons to produce leucine-requiring proteins. As these cells may have selective advantage over wild organisms due to virus-resistance, we also repurpose a third codon to biocontain this virus-resistant host via dependence on an amino acid not found in nature¹⁰. Our results suggest a general strategy to make any organism safely resistant to all natural viruses and prevent genetic information flow into and out of genetically modified organisms.

The genetic code allows organisms to exchange functions through horizontal gene transfer (HGT) and enables recombinant gene expression in heterologous hosts. However, the shared language of the same code permits the undesired spread of antibiotic, herbicide, and pesticide-resistance genes and allows viruses to cause diseases. By exploiting the shared nature of the genetic code, recombinant DNA technologies revolutionized our ability to produce small molecules, peptides, biologics, and enzymes in vast quantities; however, the production cell cultures remained susceptible to viral contamination. To date, viral contamination in cell cultures exists as a real risk with severe consequences: Over the past four decades, dozens of viral contamination cases were documented in industry^{11–13}. Horizontal gene transfer also threatens the safe use of Genetically Modified Organisms (GMOs) by enabling the spread of their engineered genetic information into natural ecosystems. Despite the impact of viral infections, HGT and the growing economic and societal role of GMOs and recombinant DNA, to date, no technology exists that could prevent viral infections and the escape of engineered genetic information from genetically modified biological systems. It is widely hypothesized that Genomically Recoded Organisms (GROs), whose genomes have been systematically redesigned to confer an alternate genetic code, would offer genetic isolation from natural ecosystems by obstructing the translation of horizontally transferred genetic material^{1–5,14}, including both resistance to viral infections and horizontal gene transfer. Indeed, the genome-wide removal of TAG stop codons and release factor 1 (RF1) from *E. coli*, which abolishes cells' ability to terminate translation at TAG stop codons, provides substantial but not complete resistance to bacteriophages^{2,3}. Most recently, a strain of *E. coli*, Syn61 3, was created with a synthetic recoded genome in which all annotated instances of two serine codons, TCG and TCA (together TCR), and the TGA stop codon were replaced with synonymous alternatives, and the corresponding serine tRNA genes (*serU* and *serT*) and RF1 (*prfA*) have been deleted^{4,15}. Although the compressed genetic code of Syn61 3 provided resistance to five viruses⁴, it could not prevent the escape of its engineered genetic information.

However, despite the potentially broad virus and gene transfer resistance of compressed genetic codes and these organisms' widespread industrial applicability, how natural genetic material could breach genetic-code-based resistance remained unanswered. Numerous viruses and mobile genetic elements encode parts of the translational apparatus, ranging from single tRNA genes and release factors up to lacking only ribosomal genes^{7–9}. These genes allow mobile genetic elements to reduce their dependency on host translational processes^{16–18 19–21}. Therefore, the selection pressure posed by GROs' compressed genetic

code might facilitate the rapid evolution of viruses and mobile genetic elements capable of crossing a genetic-code-based barrier. Here we show that horizontally transferred tRNA genes can readily substitute cellular tRNAs and thus abolish genetic-code-based resistance to viral infections and HGT. We provide the first example of a virus-resistant, biocontained bacterial host that prevents both incoming and outgoing horizontal gene transfer, which paves the way toward engineering multi-virus-resistant cell lines from any organisms and the safe use of GMOs in natural environments by eliminating horizontal gene transfer.

Mobile tRNAs abolish virus resistance

We first investigated whether tRNAs of mobile genetic elements can substitute cellular tRNAs and support viral infection in cells with a compressed genetic code. We sampled the mobile tRNAome, tRNA genes encoded by horizontally transferred genetic elements, by computationally screening thousands of viral genomes for the presence of tRNA genes, and then synthesizing 1192 tRNA genes from phylogenetically diverse plasmids, transposable elements, and bacteriophages infecting members of the *Enterobacteriaceae* family (Supplementary Data 1). Next, we assayed these tRNAs for their ability to produce functional tRNAs in an *E. coli* host and substitute genomic tRNA genes to translate TCR codons. As depicted in Figure 1a, this high-throughput assay is based on an *E. coli* strain with a synthetic recoded genome in which all annotated instances of two sense serine codons (TCG, TCA) and a stop codon (TAG) were replaced with synonymous alternatives, and the corresponding *serU*, *serT* tRNA genes and release factor 1 (*prfA*) have been deleted. This strain, *E. coli* Syn61⁻³, thereby relies on a 61-codon genetic code and prevents the expression of protein-coding genes containing TCR codons. Candidate tRNAs have been synthesized and cloned into a plasmid carrying each tRNA under a strong constitutive promoter together with an *nptII*_{40TCA,68TCG,104TCA,251TCG} aminoglycoside-O-phosphotransferase antibiotic resistance gene containing TCA codons at positions 40, 104 and TCG codons at positions 68 and 251. In wild-type *E. coli* cells bearing the canonical genetic code, *nptII*_{40TCA,68TCG,104TCA,251TCG} confers resistance to kanamycin through serine incorporation at positions 40, 68, 104, and 251, and the production of full-length aph(3')-II aminoglycoside-O-phosphotransferase. In Syn61⁻³, however, the production of this resistance-conferring gene product is inhibited due to the lack of *serU* and *serT*-encoded tRNA-Ser_{UGA} and -Ser_{CGA} needed for TCR codon decoding. Therefore, in our screen, only plasmid variants that are expressing tRNAs capable of decoding TCR codons will survive kanamycin selection. The transformation of this plasmid library into Syn61⁻³ and subsequent selection in the presence of kanamycin yielded thousands of colonies, indicating the presence of TCR translating tRNAs in our library. Pooled extraction of plasmid variants from kanamycin-resistant colonies followed by amplicon sequencing of their tRNA-insert identified 62 tRNA sequences capable of promoting *nptII*_{40TCA,68TCG,104TCA,251TCG} expression (Figure 1b, Supplementary Data 1). These tRNAs represent 89% of all predicted TCR codon-recognizing tRNAs in our library and share 33.7–61.1% (median = 46.2%) similarity to the endogenous *serU* tRNA of *E. coli*. In agreement with the anticodon composition of mobile Ser tRNAs, most tRNA hits contained a UGA anticodon and carried the identity elements necessary for recognition by the host's SerS serine-tRNA-ligase (Figure 1b).

Notable examples include the UAG anticodon-containing serine tRNA of the laboratory model coliphage T5, tRNAs from plasmids of multidrug-resistant *E. coli* isolates (GenBank IDs [AP018804](#) and [CP023851](#)), and the Ser-tRNA_{CGA} of the integrative conjugative element of *Acidithiobacillus ferrooxidans*. The presence of mobile tRNAs in integrative conjugative elements is especially concerning as these mobile genetic elements can carry up to 38 tRNAs corresponding to all 20 amino acids in a single operon and are capable of excision and transfer into neighboring bacterial cells^{20,22}. In agreement with prior studies^{7,8,23}, our computational screen also showed that mobile tRNA genes are not limited to mobile genetic elements of bacteria. Computational analysis of viruses infecting *Vertebrates* and *Archaea* revealed the presence of sense and stop codon suppressor tRNAs in both groups, suggesting that mobile tRNAs are prevalent across viruses infecting prokaryotic, archaeal, and eukaryotic hosts (Supplementary Data 2).

We confirmed the TCR codon-recognizing tRNAs' predicted serine amino acid identity by coexpressing a selected tRNA hit with an elastin_{16TCA}-sfGFP-His6 construct harboring a single TCA codon at position 16. The coexpression of the tRNA-Ser_{UGA} of *Escherichia* phage IrisVonRoten²⁴ together with the elastin_{16TCA}-sfGFP-His6 construct conferred near wild-type level expression (Figure 1c) and tryptic digest followed by reverse-phase liquid chromatography and tandem mass spectrometry (LC/MS-MS) confirmed serine incorporation at the TCA position (Extended Data Figure 1a).

Next, we investigated whether mobile tRNA_{ome}-derived tRNAs could promote viral replication. A previous study demonstrated that Syn61-3 resists infection by multiple bacteriophages, including Enterobacteria phage T6⁴. Infecting Syn61-3 with T6 phage recapitulated these results. In contrast, the infection of Syn61-3 harboring a bacteriophage-derived Ser-tRNA_{UGA} gene with T6 resulted in rapid lysis, indicating that tRNA genes that reside in viral genomes can substitute cellular tRNAs and promote phage infection (Figure 1d).

The discovery of diverse TCR codon translating tRNAs on horizontally transferred genetic elements indicates that mobile tRNA genes are widespread and can readily complement the lack of cellular tRNAs to promote viral replication and horizontal gene transfer.

Viruses infecting a recoded organism

We next investigated whether lytic viruses of Syn61-3 exist. We infected Syn61-3 cells with eleven coliphages whose genome harbor TCR translating tRNA genes based on our plasmid-based screen (Figure 1b). Surprisingly, none of these eleven phages could overcome the recoded host's genetic isolation, indicating that the presence of tRNA genes on viral genomes does not directly rescue viral replication in recoded organisms (Extended Data Figure 1b).

We next attempted to isolate lytic viruses from diverse environmental samples by performing a standard two-step enrichment-based phage isolation protocol and using Syn61-3 as host. First, bacteria-free filtrates of environmental and wastewater samples ($n=13$, from Massachusetts (USA), Extended Data Table 1a) were mixed with Syn61-3

and grown until stationary phase. Next, bacterial cells were removed, and we analyzed the presence of lytic phages by mixing sample supernatants with Syn61-3 in soft-agar overlays. Five samples produced visible lysis. Viral plaque isolation from these samples followed by DNA sequencing and *de novo* genome assembly identified 12 novel phage strains. All identified phages belong to the *Caudovirales* order and the *Myoviridae* family, taxa rich in tRNA-encoding bacteriophages⁸ (Extended Data Table 1b). Computational identification of tRNA genes revealed the presence of tRNA operons in all phage isolates, with 10 to 27 tRNA genes in each genome (Supplementary Data 1). Surprisingly, all isolates harbored TCR codon translating serine tRNAs with a UGA anticodon that we identified in our earlier *nptIII*_{40TCA,68TCG,104TCA,251TCG} suppressor screen (Figure 1b). One isolate, REP1, also harbored a predicted homing endonuclease (Figure 2c). Homing endonucleases encoded in tRNA operons have been shown to be responsible for the horizontal transfer of tRNA gene clusters²⁵. Phage isolates showed more than two orders of magnitude difference in viral titers after replication on recoded cells (Figure 2a). One of the most virulent isolates, REP12, required 60 minutes to complete a replication cycle at 37 °C in Syn61-3 (Figure 2b).

The isolated viral strains infecting Syn61-3 show that bacteriophages that can overcome sense codon recoding-based viral resistance exist and are widespread in environmental samples. However, the presence of TCR codon translating serine tRNAs on viral genomes does not directly rescue viral replication in the recoded strain.

Viral tRNAs substitute cellular tRNAs

We next investigated how tRNA-encoding viruses evade genetic-code-based resistance. Time-course transcriptome analysis of REP12 phage-infected Syn61-3 cells during the viral replication cycle revealed early and high-level expression of the viral tRNA operon (Supplementary Figure 1). In agreement with this observation, the computational prediction of bacterial promoters driving the tRNA array indicated the presence of multiple strong constitutive promoters upstream of the tRNA operon region. We then investigated the time-course kinetics of tRNA expression in Syn61-3 cells that were infected with our REP12 phage by performing tRNA sequencing (tRNAseq). Time-course tRNAseq experiments revealed remarkably high-level expression of the viral tRNA-Ser_{UGA} immediately after phage attachment (*i.e.*, a relative viral tRNA-Ser_{UGA} abundance of 56.1% ($\pm 5\%$) compared to the host *serV* tRNA) (Extended Data Figure 2). Throughout the entire phage replication cycle, the phage tRNA-Ser_{UGA} remained one of the most abundant viral tRNA species inside infected Syn61-3 cells (Extended Data Figure 2). We next investigated whether phage tRNA-Ser_{UGA} participates in translation by analyzing the presence of their mature form. The gene encoding the tRNA-Ser_{UGA} in REP12's genome does not encode the universal 5'-CCA tRNA tail, which allows for amino acid attachment as well as for interaction with the ribosome. Therefore, CCA tail addition must happen before these tRNAs can participate in translational processes. The sequencing-based analysis of phage tRNA-Ser_{UGA} ends detected CCA tail addition in 62.9% ($\pm 1.9\%$) of all tRNA sequencing reads immediately after phage attachment, indicating that mature tRNA-Ser_{UGA}s are instantly produced after host infection (Extended Data Figure 3).

We also investigated transcriptomic changes in Syn61-3 during phage replication. Analysis of the host transcriptome after phage infection revealed upregulation in genes responsible for tRNA maturation and modification. Upregulated genes include *queG*, encoding epoxyqueuosine reductase that catalyzes the final step in the *de novo* synthesis of queuosine in tRNAs²⁶, and *trmJ*, tRNA^{Cm32/U^m32} methyltransferase²⁷, which introduces methyl groups at the 2'-O position of U³² of several tRNAs, including tRNA-Ser_{UGA}, suggesting the potential posttranscriptional modification of phage-derived tRNAs (Extended Data Figure 4).

Finally, we also validated the role of phage tRNA-Ser_{UGA} tRNAs in decoding TCR codons. We first cloned the REP12 viral tRNA operon containing the hypothetical tRNA-Ser_{UGA} and its predicted promoter into a plasmid vector. Coexpression of this tRNA operon with an elastin₁₆ TCA-sfGFP-His6 and elastin₁₆ TCG-sfGFP-His6 construct, harboring either a single TCA or TCG codon at position 16, respectively, resulted in high-level elastin-sfGFP-His6 expression (Figure 2d). Next, tryptic digestion followed by LC/MS-MS analysis confirmed serine incorporation in response to both the TCA and TCG codon in these elastin₁₆ TCR-sfGFP-His6 samples (Figure 2e, Supplementary Figure 2). As expected, the coexpression of the same elastin₁₆ TCA-sfGFP-His6 construct with the only tRNA-Ser_{UGA} of the viral tRNA operon conferred a similar effect, and LC/MS-MS analysis confirmed the role of this tRNA in decoding viral TCR codons as serine (Supplementary Figure 2).

Together these results show that lytic phages of Syn61-3 overcome genetic-code-based viral resistance by rapidly complementing the cellular tRNA pool with virus-encoded tRNAs.

Swapped genetic code resists viruses

We predicted that establishing an artificial genetic code, in which TCR codons encode an amino acid different from their natural serine identity, would create a genetic firewall that safeguards cells from horizontal gene transfer and infection by tRNA-encoding viruses. In an amino-acid-swapped genetic code, viral tRNAs would compete with host-expressed tRNAs that decode TCR codons as a non-serine amino acid resulting in the mistranslation of viral proteins. As the genetic isolation provided by amino-acid-swapped genetic codes is expected to correlate with the hydrophobicity and polarity distance between exchanged amino acids²⁸, we sought to reassign TCR serine codons to leucine, the amino acid most distant from TCR codons' natural serine meaning. The more distant Trp, Phe, Tyr, and Ile exchanges were not investigated as their cognate aminoacyl-tRNA synthetases are nonpermissive to anticodon mutations and expected to require extensive enzyme and/or tRNA engineering to function²⁹⁻³¹. Although alternative forms of swapped codes emerged in the past, these codes either targeted the exchange of chemically more similar amino acids (*i.e.*, Ser to Ala, His, and Pro) or remained tested *in vitro* only^{6,28,32,33}.

To establish a serine_{TCR}-to-leucine swapped genetic code (Figure 3a), we utilized Syn61-3, which genome-wide lacks annotated instances of TCR codons and their corresponding tRNA genes, and sought to identify tRNAs capable of efficiently translating TCR codons as leucine. We modified our high throughput tRNA library screen (Figure 1a) to evolve TCR suppressors from the endogenous *E. coli leuU* tRNA carrying a TCA and TCG decoding

anticodon. We coexpressed a 65,536-member mutagenized library of the anticodon-swapped *leuU* tRNA gene in which the anticodon loop of both tRNAs has been fully randomized, together with an *aph3Ia*_{29×Leu→TCR}, a kanamycin resistance-conferring gene in which all 29 instances of leucine codons were replaced with TCR serine codons. In this system, only anticodon-swapped *leuU* variants capable of simultaneously translating all 29 TCR codons as leucine would confer resistance to kanamycin. High kanamycin selection pressure in combination with 29 instances of TCR codons in *aph3Ia* was expected to select tRNA variants that provide wild-type level translation efficiency for TCR codons. We identified two distinct *leuU* variants by applying “high” kanamycin concentration (*i.e.*, 200 µg/ml) as selection pressure to Syn61-3 cells carrying the anticodon-swapped tRNA library. These variants, carrying tRNAs containing distinct anticodon loop mutations (Extended Data Figure 5a), were then infected with a cocktail of all twelve phage isolates (Extended Data Table 1) that are capable of lysing Syn61-3 at a 10:1 cell-to-phage ratio (*i.e.*, a Multiplicity of Infection (MOI) of 0.1). Instead of plaque and efficiency-of-plating assays^{34,35} with selected phage isolates and assaying viral titer on the resistant strain, we quantified virus resistance using a mixture of all twelve Syn61-3-infecting viral isolates, and measured virion release from infected cells after 24 hours by plating culture-supernatants on a virus-susceptible wild-type *E. coli* host. This can reveal even slow or non-plaque-forming viral replication in the target cells^{35,36} (Supplementary Note).

Surprisingly, all selected *leuU* library members allowed robust phage replication, with phage titers reaching ~10⁷ PFU/ml after 24 hours (Figure 3c). We hypothesized that viral replication in the presence of TCR suppressing *leuU* variants is due to these tRNAs lower suppression efficiency compared to competing phage-carried serine tRNAs, which leads to rapid viral takeover. Viral tRNA-Ser_{YGA}, that are tRNA-Ser_{UGA} and tRNA-Ser_{CGA}, might have i.) higher aminoacylation efficiency by their corresponding *E. coli* aminoacyl-tRNA-ligase than our selected *leuU* variants, ii.) higher affinity towards the bacterial ribosome, and/or iii.) better evade phage- and host-carried tRNA-degrading effector proteins^{16,37,38}.

Based on this observation, we hypothesized that bacteriophage-encoded tRNAs might provide much higher suppression efficiencies for their cognate codons than their native *E. coli* counterpart. Therefore, and to exploit this superior translation efficiency of bacteriophage-encoded tRNAs and their resistance to phage-carried tRNA degrading enzymes, we next constructed a small, focused library that coexpressed YGA anticodon-swapped mutants of 13 phage-encoded leucine tRNAs, together with the *aph3Ia*_{29×Leu→TCR} aminoglycoside O-phosphotransferase gene. The transformation of this library into Syn61-3 cells and subsequent “high” concentration (*i.e.*, 200 µg/ml) kanamycin selection identified three distinct tRNAs enabling robust growth. Identified tRNAs showed only 48.3–37.9% similarity to *E. coli leuU* but carried most of the canonical *E. coli* leucine-tRNA ligase identity elements (Extended Data Figure 5b). Furthermore, the analysis of the total tRNA content of these cells by tRNAseq confirmed the presence of synthetic phage Leu-tRNA_{YGA} tRNAs with similar abundances as the cellular endogenous serine tRNAs (*i.e.*, a relative expression level of 172% and 140% for Leu-tRNA_{UGA} and Leu-tRNA_{CGA} respectively, compared to *serV* (Extended Data Figure 6)).

Next, similarly to our previous infection assay, phage tRNA_{YGA} expressing cells were infected with a mixture of twelve distinct, lytic phages of Syn61-3 at a MOI = 0.1. The analysis of phage titer in culture supernatants after 24 hours showed a marked drop compared to the input phage inoculum, suggesting that anticodon-swapped viral leucine tRNAs block phage replication (Figure 3c).

We then investigated the mechanism of phage resistance in *E. coli* cells carrying virus-derived tRNA-Leu_{YGA} tRNAs (Ec_Syn61-3 Ser→Leu Swap, or Ec_Syn61-3-SL in short, Figure 3b) by performing total proteome analysis. Untargeted deep proteome analysis of uninfected cells by tandem mass spectrometry validated the translation of TCR codons as leucine in Ec_Syn61-3-SL (Figure 3d). Time-course untargeted proteome analysis after bacteriophage infection revealed extensive mistranslation at TCR codons in newly synthesized phage proteins (Figure 3e, Supplementary Figure 3), indicating that an amino-acid-swapped genetic code broadly obstructs viral protein synthesis. In agreement with earlier reports that showed the partial recognition of TCT codons by tRNA_{UGA}^{39,40} and due to the extreme sensitivity of our untargeted proteomics assay, we also detected serine-to-leucine mistranslation at TCT codon positions in Ec_Syn61-3-SL cells (Extended Data Figure 7). The recognition of TCT codons by phage tRNA-Leu_{YGA} tRNAs might also be responsible for the fitness decrease of Ec_Syn61-3-SL cells compared to its ancestor strain (*i.e.*, a doubling time of 69.3 minutes, compared to 44.29 minutes for the parental Syn61-3 strain in rich 2×YT media (Extended Data Figure 8)). Alternatively, the fitness decrease of Ec_Syn61-3-SL might also be attributable to the presence of TCR codons in essential genes of Syn61-3. According to our genome analysis, at least four essential genes of Syn61-3, *mukE*, *ykfM*, *yjbS*, *safA*, contain TCR codons and become mistranslated in Ec_Syn61-3-SL (Supplementary Data 4).

Finally, we also sought to develop a tightly biocontained version of Ec_Syn61-3-SL because a virus-resistant strain might have a competitive advantage in natural ecosystems due to the lack of predating bacteriophages. Synthetic auxotrophy based on the engineered reliance of essential proteins on human-provided nonstandard amino acids (nsAAs), *e.g.*, L-4,4'-biphenylalanine (bipA), offers tight biocontainment that remains stable under long-term evolution^{10,41,42}. Therefore, we generated a recombination deficient (*i.e.*, *recA*), biocontained version of Ec_Syn61-3-SL bearing a bipA-dependent essential *adk* gene and the bipA aminoacyl-tRNA synthetase/tRNA-bipA_{CUA} system, by first performing adaptive laboratory evolution on a *recA* knock-out Syn61-3, and then replacing the genomic *adk* copy with its bipA-dependent variant⁴³ (Methods). This strain maintained the low escape frequency of previously reported single-gene synthetic auxotrophs¹⁰ (*i.e.*, 2.9×10^{-6} ($\pm 5.9 \times 10^{-7}$) escape frequency) and provided robust growth. We also tested the viral resistance of Ec_Syn61-3-SL under mock environmental conditions by repeating our phage enrichment and isolation process with a mixture of 12 environmental samples, including fresh sewage (Extended Data Table 1, Supplementary Note), but could not detect plaque-forming phages in culture supernatants (Extended Data Figure 9).

Together, these results demonstrate that reassigning sense codons TCA and TCG to leucine *in vivo* provides broad protection against viruses, including novel mixtures of viruses directly from environmental samples, and the TAG stop codon can be simultaneously

utilized to biocontain this virus-resistant strain via dependence on an amino acid not found in nature.

Genetic-code-based bidirectional firewall

Finally, we developed a set of plasmid vectors that we systematically addicted to an amino-acid-swapped genetic code in which leucine is encoded as TCR codons. GMOs are increasingly deployed for large-scale use in agriculture, therapeutics, bioenergy, and bioremediation. Consequently, it is critical to implement robust biocontainment strategies that prevent the unintended proliferation of GMOs and protect natural ecosystems from engineered genetic information. Although efficient biocontainment strategies exist (*e.g.*, bipA nsAA-based synthetic auxotrophy, as in Ec_Syn61³-SL), current methods fail to prevent the horizontal gene transfer (HGT)-based escape of engineered genetic information. Synthetic addiction to a swapped genetic code offers a solution to this problem. Using our virus-derived tRNA-Leu_{YGA} expressing Ec_Syn61³-SL cells, we, therefore, developed a set of plasmid vectors that depend on TCR codons to express leucine-containing proteins and thus can only function in cells that efficiently translate TCR codons as leucine (Figure 4a). These plasmids, called the pLS plasmids, offer four widely used, orthogonal antibiotic resistance markers in combination with four mutually orthogonal low- to high-copy-number origins-of-replication for stable maintenance in Ec_Syn61³-SL cells (Figure 4b, Supplementary Data 3). Antibiotic resistance genes and proteins necessary for pLS plasmid replication encode leucine as TCR—naturally serine-meaning—codons and, therefore, fail to function in cells bearing the canonical genetic code. The addition of resistance markers and replication proteins to an artificial genetic code ensures that pLS plasmids can stably and safely maintain synthetic genetic functions but restrict these genes' functionality to Ec_Syn61³-SL cells. Using our pLS plasmids, we achieved serine-to-leucine codon reassignment simultaneously at ~14,000 codon positions in a single cell, thus demonstrating our viral tRNA-based system's exceptional codon reassignment efficiency.

We then tested the ability of our pLS vectors to function in cells bearing the standard genetic code cells by electroporating plasmid DNA from six variants (*i.e.*, pLS1–6) into wild-type *E. coli* K-12 MG1655 cells but could not detect escapees carrying pLS plasmids (*i.e.*, an electroporation efficiency of <1 CFU/μg, while the electroporation efficiency the recipients cells was $3 \times 10^9 \pm 1.1 \times 10^9$ CFU/μg (\pm s.d., $n = 3$ independent experiments) based on a pUC-Kan^R control plasmid). The escape of pLS plasmids was similarly prevented when the phage tRNA-Leu_{YGA} expression cassette was encoded within the plasmid backbone (*i.e.*, pLS1 and pLS2), indicating that anticodon-swapped viral tRNAs are severely toxic to wild-type cells. Based on these results, we also expect that, similarly to pLS' genes, any leucine-requiring protein, up to entire chromosomes, can be addicted to Ec_Syn61³-SL by recoding target genes to encode one or more leucine positions as TCR codons.

In sum, the addition of pLS plasmids to an artificial genetic code in which leucine is encoded as TCR codons, in combination with nsAA-based synthetic auxotrophy, promises escape-free biocontainment for engineered genetic information.

Discussion

Previous works provided support for using rational genetic code engineering to isolate GMOs from natural ecosystems by preventing viral infections and horizontal gene transfer²⁻⁴; however, how natural viruses and transferred genes could breach genetic-code-based resistance remained unanswered. By systematically investigating horizontal gene transfer into *E. coli* Syn61-3, a synthetic organism with a compressed genetic code⁴, we demonstrated that tRNAs expressed by bacteriophages, plasmids, and integrative conjugative elements, readily substitute cellular tRNAs and abolish genetic-code-based isolation. We discovered twelve viruses in environmental samples that can infect *E. coli* Syn61-3, despite its compressed genetic code (Figure 2a). These bacteriophages express up to 27 tRNAs, including a functional tRNA-Ser_{UGA} needed to replace the host's deleted tRNAs. These findings suggest that the selection pressure posed by compressed genetic codes can facilitate the rapid evolution of viruses and mobile genetic elements capable of crossing a genetic-code-based barrier. This hypothesis is further supported by the co-localization of tRNAs and homing endonucleases (Figure 2c) that catalyze copy-and-paste tRNA operon transfer²⁵. We have also shown that mobile tRNAs are not limited to bacteria, as multiple archaeal and eukaryotic viruses also carry predicted tRNA genes.

Next, as a combined solution against horizontal gene transfer and viral infections, including tRNA-expressing viruses, we have created a new type of biocontained *E. coli* strain, Ec_Syn61-3-SL, carrying an amino-acid-swapped genetic code. Ec_Syn61-3-SL achieves unprecedented gene-transfer resistance by the reassignment of TCR codons to leucine—an amino acid distant in physicochemical properties compared to their natural serine identity—using reprogrammed, virus-derived tRNAs. In contrast to prior studies^{2-4,33}, we confirmed the virus resistance of this strain using a broad range of viruses. We found that viral tRNAs provide exceptional codon reassignment efficiency and are superior for establishing altered genetic codes compared to cellular tRNAs. We also showed that the swapped genetic code of Ec_Syn61-3-SL simultaneously prevents viral replication and the escape of synthetic genetic information into wild organisms. As a direct application, we addicted the most widely used plasmid vectors to express leucine-containing proteins with TCR codons and developed the first plasmids that cannot function in natural organisms. By demonstrating efficient serine-to-leucine reassignment at a previously unprecedented scale, we made biological containment possible for genes, operons, up to entire chromosomes. Adaptive laboratory evolution, that have significantly improved doubling times of organisms with altered genetic codes^{4,44}, will be applied to Ec_Syn61-3-SL to help move toward industrial use.

Potential limitations of our work include the sampled viral diversity and the inability of our viral resistance assays to sample multi-step, long-term evolutionary processes of viruses. Although our tests of Ec_Syn61-3-SL's viral resistance included twelve lytic Syn61-3 phages and a complex mixture of environmental samples, including fresh sewage, we cannot rule out the existence of Ec_Syn61-3-SL-infecting phages in Earth's biome. Our assays provided an example of Ec_Syn61-3-SL culture contamination with soil, sewage, and fecal material (Extended Data Table 1a); however, lysogenic viruses or jumbo- and megaphages, with genomes of more than 200 and 500 kbp, respectively, could have remained undetected

in our assays due to their frequent inability to form visible plaques^{9,36}. We hypothesize that bacteriophages with few TCR codons in essential genes, *e.g.*, *Escherichia phage EC6098* with only 33 TCR positions in its six protein-coding genes⁴⁵, have the highest potential to overcome an amino-acid-swapped code. Alternatively, viral tRNA-degrading proteins^{37,38} could evolve to selectively destroy mistranslating tRNAs and thus promote viral escape; however, both mechanisms would require multiple simultaneous mutations and thus unlikely to occur. Future work will explore whether such viruses can evade the amino-acid-swapped genetic code of Ec_Syn61³-SL.

After the publication of our preprint⁴⁶, Zürcher et al.³³ confirmed some of these findings by identifying two tRNAs that could overcome Syn61³'s virus resistance and by noting the utility of swapped genetic codes to obstruct HGT. The use of weak *E. coli* tRNAs in our hands was insufficient to produce true resistance to a broad set of viruses. The apparent success with such weak tRNAs in Zürcher et al. may be due to testing only two (related) viral strains and using efficiency-of-plating^{34,35} and conjugation assays (Supplementary Note). Furthermore, the lower expression level of mistranslating tRNAs used by Zürcher et al., in combination with the use of *E. coli* tRNAs still susceptible to viral tRNA-degrading enzymes^{37,38}, might result in compromised resistance in the presence of complex environmental viral communities. Zürcher et al. did not implement biocontainment strategies to limit the unwanted proliferation of their potentially virus-resistant host.

These findings fundamentally impact ongoing prokaryotic and eukaryotic genome recoding projects^{5,47-49}, including our aim to engineer a virus-resistant stop-codon-recoded human cell line and a 57-codon strain of *E. coli*, as some of the identified tRNAs are expected to enable viral replication in these recoded organisms. Therefore, we are now implementing the swapped genetic code of this work to ensure the virus and HGT resistance of these engineered hosts. We expect that our results will have implications for the safe use of GMOs in open environments by establishing a generalizable method for genetic code alteration that simultaneously prevents viral predation in natural ecosystems and blocks incoming and outgoing HGT with natural organisms. Follow-up works could utilize biocontained swapped genetic codes to prevent transgene release during bioremediation, offer containment for open cultures of engineered photoautotrophs for carbon sequestration, and enable safe microbiome engineering and vaccine production by addicting living treatments to use a swapped code. The combination of genome recoding and codon swap may provide a universal strategy to make any species resistant to many or all natural viruses.

Methods

Bacterial media and reagents

Lysogeny Broth Lennox (LBL) was prepared by dissolving 10 g/l tryptone, 5 g/l yeast extract, and 5 g/l sodium chloride in deionized H₂O and sterilized by autoclaving. Super Optimal Broth (SOB) was prepared by dissolving 20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l sodium chloride, 2.4 g/l magnesium sulfate, and 0.186 g/l potassium chloride in deionized H₂O and sterilized by autoclaving. 2×YT media consisted of 16 g/l casein digest peptone, 10 g/l yeast extract, 5 g/l sodium chloride. LBL and 2×YT agar plates were prepared by supplementing LBL medium or 2×YT with agar at 1.6% w/v before autoclaving. Top

agar for agar overlay assays was prepared by supplementing LBL medium with agarose at 0.7% w/v before autoclaving. SM Buffer, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM MgSO₄, 0.01% gelatin, was used for storing and diluting bacteriophage stocks (Geno Technology, Inc., St. Louis, MO, USA). L-4,4'-biphenylalanine (bipA) was obtained from PepTech Corporation (USA).

Bacteriophage isolation

Bacteriophages were isolated from environmental samples from Massachusetts, United States, collected in Q3 2021 (Samples 1–2, 4–13) and Q1 2022 (Samples 1–3, Extended Data Table 1a), and by using *E. coli* Syn61 3(ev5) (from the laboratory of Jason W. Chin (Addgene strain #174514)) as host. For aqueous samples, including sewage, we directly used 50 ml filter-sterilized filtrates, while samples with mainly solid components, like soil and animal feces, were first resuspended to release phage particles and then sterilized by centrifugation and subsequent filtration. Environmental samples and the sterilized filtrates were stored at 4 °C in the dark. This protocol avoided the inactivation of chloroform-sensitive viruses. Sterilized samples were then mixed with exponentially growing cultures of Syn61 3(ev5) in SOB supplemented with 10 mM CaCl₂ and MgCl₂. Infected cultures were grown overnight at 37 °C aerobically and then filter sterilized by centrifugation at 4000× g for 15 minutes and filtered through a 0.45 µm PVDF Steriflip™ disposable vacuum filter unit (MilliporeSigma). Next, 1 ml from each sterilized enriched culture was mixed with 10 ml exponentially growing Syn61 3 (OD₆₀₀ = 0.2), supplemented with 10 mM CaCl₂ and MgCl₂, and mixed with 10 ml 0.7% LBL top agar. Top agar suspensions were then poured on top of LBL agar plates in 145×20 mm Petri dishes (Greiner Bio-One). Petri dishes were incubated overnight at 37 °C and inspected for phage plaques on the next day. Areas with visible lysis or plaques were excised, resuspended in SM buffer, and diluted to single plaques on top agar lawns containing 99% Syn61 3 and 1% MDS42 cells. We note that adding trace amounts of MDS42 cells increased the visibility of plaques, and clear plaques, indicating phage replication on the recoded host, could be easily picked. Dilutions and single plaque isolations were repeated four times for each plaque to purify isogenic phages. Finally, high-titer stocks were prepared by mixing sterilized suspensions from single plaques with exponentially growing MDS42 cells (OD₆₀₀ = 0.3) in SOB supplemented with 10 mM CaCl₂ and MgCl₂. Phage-infected samples were grown at 37 °C until complete lysis (~4 hrs) and then sterilized by filtration.

Bacteriophage culturing

Bacteriophage stocks were prepared by a modified liquid lysate Phages on Tap protocol in LBL medium⁵¹. High-titer lysates were prepared from single plaques by picking well-isolated phage plaques into SM buffer and then seeding 3–50 ml early exponential phase cultures of *E. coli* MDS42 cells with the resulted phage suspension in SOB supplemented with 10 mM CaCl₂ and MgCl₂. Phage infected samples were grown at 37 °C until complete lysis and then sterilized by filtration. High-titer phage lysates were stored at 4 °C in the dark. Phages were archived as virocells and stored at –80 °C in the presence of 25% glycerol for long-term storage.

Phage replication assay

Genomic TCR-suppressor tRNA-Ser_{UGA} gene containing phages (based on Supplementary Data 1), corresponding to NCBI GenBank numbers [MZ501046](#), [MZ501058](#), [MZ501065](#), [MZ501066](#), [MZ501067](#), [MZ501074](#), [MZ501075](#), [MZ501089](#), [MZ501096](#), [MZ501098](#), [MZ501105](#), [MZ501106](#)²⁴, were obtained from DSMZ (Germany). Exponential phase cultures (OD₆₀₀ = 0.3) of MDS42 and Syn61₃(ev5) were grown in SOB supplemented with 10 mM CaCl₂ and MgCl₂ at 37 °C. Cultures were infected with phage at an MOI of approximately 0.001. Simultaneously, the same amount of each phage was added to sterile SOB supplemented with 10 mM CaCl₂ and MgCl₂ to act as a cell-free control for input phage calculation. Replication assays with the T6 bacteriophage in Syn61₃ on Figure 1d were performed by infecting exponential phase cultures at an MOI of 0.01 with T6 phage. The figure shows total T6 titer after 24 hours of incubation. Infected cultures were grown at 37 °C with shaking at 250 rpm. After 24 hours, cultures were transferred to 1 ml tubes and centrifuged at 19,000x g to remove cells and cellular debris, and the clarified supernatant was serially diluted in SM buffer to enumerate output phage concentration. 1.5 µl of the diluted supernatants were applied to LBL 0.7% top agar seeded with MDS42 cells and 10 mM CaCl₂ and MgCl₂ using a 96 fixed pin multi-blot replicator (VP407, V&P Scientific). Following 18 hours of incubation at 37 °C, plaques were counted, and the number of plaques was multiplied by the dilution to calculate the phage titer of the original sample.

Single-step phage growth curve

An exponential phase culture (OD₆₀₀ = 0.3) of Syn61₃ was grown in 50 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂ at 37 °C with shaking at 250 rpm. Cultures were then spun down and resuspended in 3 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂, and 1 ml samples were infected with REP12 phage at an MOI of 0.01. Infected cultures were incubated at 37 °C for 10 minutes without shaking for phage attachment and then washed twice with 1 ml SOB by pelleting cells at 4000x g for 3 minutes. Infected cells were then diluted into 50 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂ and incubated at 37 °C with shaking at 250 rpm. At every 20 minutes, 1 ml sample was measured out into a sterile Eppendorf tube containing 100 µl chloroform, immediately vortexed, and then placed on ice. Phage titers were determined by centrifuging chloroformed cultures at 6000x g for 3 minutes and then serially diluting supernatants in SM buffer and spotting 1 µl dilutions to LBL 0.7% top agar plates seeded with MDS42 cells and 10 mM CaCl₂ and MgCl₂. Following 18 hours of incubation at 37 °C, plaques were counted, and the number of plaques was multiplied by the dilution to calculate the phage titer of the original sample.

Bacteriophage genome sequencing, assembly, and annotation

Genomic DNA of bacteriophages was prepared from high-titer (*i.e.*, >10¹⁰ PFU/mL) stocks after DNase treatment using the Norgen Biotek Phage DNA Isolation Kit (Cat# 46800) according to the manufacturer's guidelines and sequenced at the Microbial Genome Sequencing Center (MiGS, Pittsburgh, PA, USA). Sequencing libraries were prepared using the Illumina DNA Prep kit and IDT 10 bp UDI indices and sequenced on an Illumina NextSeq 2000, producing 150 bp paired-end reads. Demultiplexing, quality control, and

adapter trimming were performed with bcl-convert (v3.9.3). Reads were trimmed to Q28 using BBDuk from BBTools. Phage genomes were then assembled de novo using SPAdes 3.15.2 in --careful mode with an average read coverage of 10–50×. Assembled genomes were then annotated using Prokka version 1.14.6⁵² with default parameters, except that the PHROGs HMM database⁵³ was used as input to improve phage functional gene annotations.

Bacterial genome sequencing and annotation

Genomic DNA from overnight saturated cultures of isogenic bacterial clones was prepared using the MasterPure™ Complete DNA and RNA Purification Kit (Lucigen) according to the manufacturer's guidelines and sequenced at the Microbial Genome Sequencing Center (MiGS, Pittsburgh, PA, USA). Sequencing libraries were prepared using the Illumina DNA Prep kit and IDT 10 bp UDI indices and sequenced on an Illumina NextSeq 2000, producing 150 bp paired-end reads. Demultiplexing, quality control, and adapter trimming were performed with bcl-convert (v3.9.3). Reads were then trimmed to Q28 using BBDuk from BBTools and aligned to their corresponding reference by using Bowtie2 2.3.0⁵⁴ in --sensitive-local mode. Single-nucleotide polymorphisms (SNPs) and indels were called using breseq (version 0.36.1)⁵⁵. Only variants with a prevalence higher than 75% were voted as mutations. Following variant calling, mutations were also manually inspected within the aligned sequencing reads in all cases.

The *de novo* sequencing and genome assembly of Syn61 3(ev5) (from a single-colony isolate of Addgene strain #174514) was performed by generating 84,136 Oxford Nanopore (ONT) long-reads by PCR-free library generation (Oxford Nanopore, UK) on a MinION Flow Cell (R9.4.1) and 4.5×10^6 150 bp paired-end reads on an Illumina NextSeq 2000. Quality control and adapter trimming were performed with bcl2fastq 2.20.0.445 and porechop 0.2.3_seqan2.1.1 for Illumina and ONT sequencing, respectively. Next, we performed hybrid assembly with Illumina and ONT reads by using Unicycler 0.4.8 by using the default parameters. Finally, the resulted single, circular contig representing the entire genome was manually inspected for errors in Geneious Prime® 2022.1.1. and annotated based on sequence homology by using the BLAST function (version 2.11.0) implemented in Geneious Prime® 2022.1.1. based on *E. coli* K-12 MG1655 (NCBI ID: U00096.3) as reference. Gene essentiality was determined based on Ref⁵⁶.

Transcriptome analysis of phage-infected cells

We explored transcriptomic changes and mRNA production in phage-infected Syn61 3 cells by performing a modified single-step growth experiment and collected samples at 20 minutes intervals. 50 ml of early-exponential ($OD_{600} = 0.15$) Syn61 3 cells (corresponding to 2×10^{10} CFU) growing at 37 °C, 250 rpm in SOB containing 10 mM CaCl₂ and MgCl₂ were spun down at room temperature and resuspended in 1 ml of SOB. 50 µl of this uninfected sample was immediately frozen in liquid N₂ and stored at –80 °C until RNA extraction. Next, 900 µl of this cell suspension was mixed with 10 ml prewarmed REP12 phage stock (*i.e.*, $\sim 7 \times 10^{10}$ PFU to achieve a MOI of ~ 4) in SOB containing 10 mM CaCl₂ and MgCl₂, and then incubated at 37 °C for 10 minutes without shaking for phage absorption. Following phage attachment, samples were spun down, washed with 1 ml SOB twice to remove unabsorbed phages, and then resuspended in 10 ml SOB containing 10

mM CaCl₂ and MgCl₂. Samples were then incubated at 37 °C, 250 rpm. After 20- and 40-minutes post-infection, we spun down 1 ml cell suspension from each sample, and the cell pellets were frozen in liquid nitrogen and stored at –80 °C until RNA extraction. As expected, after 60 minutes post-infection, no cell pellet was visible. Phage infections were performed in three independent replicates. Total RNA from frozen samples was extracted by using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions and the extracted RNA was DNase treated with Invitrogen RNase-free DNase (Thermo Fisher Scientific, USA). Sequencing library preparation was then performed using Stranded Total RNA Prep Ligation kit with Ribo-Zero Plus for rRNA depletion and by using 10 bp IDT for Illumina indices (all from Illumina, USA). Sequencing was done on a NextSeq2000 instrument in 2×50 bp paired-end mode. Demultiplexing, quality control, and adapter trimming were performed with bcl-convert (v3.9.3). cDNA reads were aligned to their corresponding reference by using Bowtie2 2.3.0⁵⁴ in --sensitive-local mode, and read count and expression metrics were determined by using Geneious Prime[®] 2022.1.1. (Biomatters Ltd.). Finally, differential expression analysis was performed by using DESeq2⁵⁰ (version 1.38.3) with default settings.

tRNA sequencing sample preparation

We explored tRNA expression levels and changes in phage-infected Syn61 3 cells by performing a modified single-step growth experiment with high MOI and cell mass. An early-exponential phase culture (OD₆₀₀ = 0.2) of Syn61 3 cells (corresponding to approximately 5×10¹⁰ CFU) growing at 37 °C, 250 rpm in SOB containing 10 mM CaCl₂ and MgCl₂ were spun down at room temperature and resuspended in 1.1 ml of SOB. 100 µl of this uninfected sample was immediately frozen in liquid N₂ and stored at –80 °C until tRNA extraction. Next, 1000 µl of this cell suspension was mixed with 20 ml prewarmed REP12 phage stock (*i.e.*, ~10¹² PFU to achieve a MOI of ~20) in SOB containing 10 mM CaCl₂ and MgCl₂, and then incubated at 37 °C for 10 minutes without shaking for phage absorption. Following phage attachment, samples were spun down, the supernatant containing unabsorbed phages was removed, and the cell pellet was then resuspended in 7 ml SOB containing 10 mM CaCl₂ and MgCl₂. Samples were then incubated at 37 °C, 250 rpm. Immediately after phage attachment and after 20- and 40-minutes post-infection, 1 ml cell suspensions from each sample were spun down, and cell pellets were frozen in liquid N₂ and stored at –80 °C until total RNA extraction. Phage infections were performed in two independent replicates.

We analyzed the total tRNA content of Ec_Syn61 3-SL cells expressing KP869110.1 viral tRNA_{24-Leu_{UGA}} and tRNA_{24-Leu_{CGA}} by pelleting cells from 5 ml mid-exponential (OD₆₀₀ = 0.3) culture at 4000× g and flash-freezing the cell pellet in liquid nitrogen.

We extracted tRNAs by lysing samples at room temperature (RT) for 30 mins in 150 µl lysis buffer containing 8 mg/mL lysozyme (from chicken egg white, #76346–678, VWR, USA), 10 mM Tris HCl pH 7.5, and 1 µl murine RNase inhibitor (New England Biolabs). Samples were then mixed with 700 µl Qiazol reagent (#79306, Qiagen) and incubated for 5 minutes at RT. Next, 150 µl chloroform was added, vortexed, and incubated until phase separation. Samples were then spun at 15,000× g for 15 min at in a cooled centrifuge. The supernatant

was transferred into a new Eppendorf tube and mixed with 350 μ l 70% ethanol. Larger RNA molecules were then bound to an RNeasy MinElute spin column (#74204, Qiagen), and the flow-through was mixed with 450 μ l of 100% ethanol, and tRNAs were bound to a new RNeasy MinElute spin column. The tRNA fraction was then washed first with 500 μ l wash buffer (#74204, Qiagen), next with 80% ethanol, and then eluted in RNase-free water. The eluted tRNAs were deacylated in 60 mM pH 9.5 borate buffer (J62154-AK, Alfa Aesar, Thermo Fisher Scientific) for 30 minutes and then purified using a Micro Bio-Spin P-30 Gel Column (7326251, from Bio-Rad).

tRNA sequencing library preparation, sequencing, and data analysis

We prepared tRNA cDNA libraries by reverse-transcribing tRNAs using the TGIRTTM-III template-switching reverse-transcriptase (TGIRT50, InGex, USA) according to the manufacturer's instructions. In brief, we prepared reaction mixtures containing 1 μ l (~100 ng) of the deacylated tRNAs, 2 μ l of 1 μ M TGIRT DNA/RNA heteroduplex (prepared by hybridizing equimolar amounts of rCrUrUrUrGrArGrCrCrUrArArUrGrCrCrUrGrArArArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrCrUrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArU/3SpC3/ and ATCGTCGGACTGTAGAACTAGACGTGTGCTCTTCCGATCTTTCAGGCATTAGGCTCAAAGN oligos), 4 μ l 5 \times TGIRTTM reaction buffer (2.25 M NaCl, 25 mM MgCl₂, 100 mM Tris-HCl, pH 7.5), 2 μ l of 100 mM DTT, 9 μ l RNase-free water, and 1 μ l TGIRT-III, and incubated at room temperature for 30 minutes to initiate template-switching. Next, 1 μ l of 25 mM dNTPs (Thermo Fisher Scientific, USA) was added to the reaction mixture, and samples were incubated at 60 °C for 30 minutes to perform reverse transcription. RNA was then hydrolyzed by NaOH, neutralized by HCl, and the cDNA library was purified using MinElute PCR purification kit. cDNAs were then ligated to a preadenylated DNA adapter/5Phos/GATCNNNAGATCGGAAGAGCGTCGTGT/3SpC3/, in which NNN denotes an N, NN, NNN spacer to increase library diversity during sequencing (preadenylated oligos were prepared by 5' DNA adenylation kit (E2610L) using thermostable 5' App DNA/RNA ligase (M0319L, both from New England Biolabs) following the manufacturer's protocol. The cDNA library was purified using the MinElute PCR purification kit (Qiagen) and amplified using Q5 Host-Start High-Fidelity 2x Master Mix (New England Biolabs). PCR products were then size selected to remove adaptor-dimers below 200 bp using three subsequent size-selection rounds with a Select-a-Size DNA Clean & Concentrator Kit (D4080, Zymo Research). Finally, amplicon libraries were barcoded using the IDT 10 bp UDI indices (Illumina) and sequenced on an Illumina MiSeq to produce 250 bp paired-end reads. Read-demultiplexing was performed with bcl-convert (v3.9.3). Paired-end reads were then aligned to their reference sequences by using Geneious assembler, implemented in Geneious Prime[®] 2022.1.1., allowing a maximum of ten SNPs within tRNA reads compared to their reference. These settings allowed us to map lower-fidelity TGIRT-III-transcribed cDNA reads to their corresponding reference sequence without cross-mapping to tRNAs sharing sequence homology. tRNA reads from Ec_Syn61 3-SL cells expressing KP869110.1 viral tRNA²⁴-Leu^{UGA} and tRNA²⁴-Leu^{CGA} were mapped without allowing the presence of SNPs in sequencing reads to distinguish tRNA²⁴-Leu^{UGA} and tRNA²⁴-Leu^{CGA} that differs by only a single SNP within the anticodon region.

Genome editing and biocontainment of Syn61 3

We first generated a deficient recombination variant of Syn61 3(ev5) by eliminating the expression of the genomic *recA* gene using Cas9-assisted recombineering. RecA deletion experiments were performed by first transforming Syn61 3(ev5) cells with a plasmid carrying a pSC101 origin-of-replication, a constitutively expressed chloramphenicol resistance marker, SpCas9 and tracrRNA (from pCas9^{57,58}, Addgene #42876), and the λ Red operon, consisting of *gam*, *exo*, and *bet* (from pORTMAGE311B⁵⁹, Addgene #120418). Next, cells were made electrocompetent using a standard protocol^{57,58} for Cas9-assisted recombineering and transformed with 2 μ l of 100 μ M 90 nucleotide-long ssDNA oligonucleotide inserting a stop codon and a frameshift mutation into *recA* (Supplementary Data 3). Successful edits were selected by cotransforming 1 μ g from a variant of the pCRISPR plasmid^{57,58} carrying a 5'-AGTTGATACCTTCGCCGTAG guide sequence to cleave the genomic *recA* sequence in unedited cells. All plasmids were recoded to lack TCR and TAG codons in protein-coding genes, and synthesized by GenScript USA Inc. The resulted Syn61 3(ev5) *recA* strain was validated by whole genome sequencing and then evolved for increased fitness (available in section “*Adaptive laboratory evolution of Syn61 3*”). Finally, the replacement of the genomic *adk* gene of Syn61 3(ev5) *recA* (ev1) with the bipA-dependent *adk.d6* variant¹⁰ was performed by first transforming cells with a plasmid carrying a constitutively expressed MjTyrRS-derived bipA aaRS (variant 10, based on Ref⁶⁰) together with its associated tRNA under the control of a *proK* tRNA promoter and an *aminoglycoside-(3)-N-acetyltransferase* gene, conferring gentamycin resistance, all on a plasmid containing a p15A origin-of-replication (Supplementary Data 3). Next, we integrated the *adk.d6* variant by Cas9-assisted recombineering as described above, but instead of oligonucleotide-mediated recombineering, we transformed 4 μ g of a dsDNA cassette carrying the full-length *adk.d6* variant with 400 bp flanking genomic homology (constructed by GenScript USA Inc., Supplementary Data 3). Cells were grown in the presence of 200 μ M bipA in 2 \times YT media throughout the entire procedure. Successful edits were selected using a dual-targeting crRNA expression construct, carrying 5'-GCAATGCGTATCATTCTGCT and 5'-GCCGTCAACTTTCGCGTATT guide sequences (from GenScript USA Inc.). Positive colonies were selected by screening colonies with allele-specific PCR (Supplementary Data 3) and validated by whole genome sequencing. Finally, the escape rate of the resulted Syn61 3(ev5) *recA* (ev1) *adk.d6* strain was determined as described earlier¹⁰, but instead of chloramphenicol, cells were grown in the presence of 10 μ g/ml gentamycin in 2 \times YT. Plates were incubated for seven days at 37 $^{\circ}$ C. Escape rate measurements were performed in triplicates; \pm indicates standard deviation.

Adaptive laboratory evolution of Syn61 3

We performed standard adaptive laboratory evolution in rich bacterial media for 30 days (~270 cell generations) on Syn61 3(ev5) *recA* cells to increase fitness. At each transfer step, 10⁹-10¹⁰ bacterial cells were transferred into 500 ml LBL medium containing 1.5 g/l Tris/Tris-HCl and incubated aerobically for 24 hours at 37 $^{\circ}$ C, 250 rpm in a 2000 ml Erlenmeyer flask with a vented cap. Following 30 transfers, bacterial cells were spread onto LBL agar plates, and an individual colony was isolated and subjected to whole-genome sequencing. The identified mutations in the resulted evolved variant, Syn61 3(ev5) *recA* (ev1), are available in Supplementary Data 3.

Doubling time measurements

To determine growth parameters under standard laboratory conditions, saturated overnight cultures of *E. coli* Syn61-3(ev5), Syn61-3(ev5) *recA*, and its evolved variant, Syn61-3(ev5) *recA* (ev1) were diluted 1:200 into 50 ml of 2×YT and LBL in a 300 ml Erlenmeyer flask with vented cap and incubated aerobically at 37 °C, 250 rpm. Ec_Syn61-3-SL cells were characterized similarly, but by using 2×YT containing 50 µg/ml kanamycin. All growth measurements were performed in triplicates. Optical density at 600 nm (OD₆₀₀) measurements were taken every 20 minutes for 8 hours or until stationary phase was reached on a CO8000 Cell Density Meter, WPA. The doubling time was calculated for each independent replicate by log₂-transforming OD₆₀₀ values and calculating the doubling time based on every six consecutive data points during the exponential growth phase. We calculated the doubling time (1/slope) from a linear fit to log₂-derivatives of the six data points within this window and reported the shortest doubling time for each independent culture. Curve fitting, linear regression, and doubling time calculations were performed with Prism9 (GraphPad). Error bars show ± standard deviation.

tRNA annotation

We detected tRNA genes in the Viral genomic NCBI Reference Sequence Database (Accessed: January 2, 2022) and in individual phage isolates' genomes by using tRNAscan-SE 2.0.9 in bacterial (-B), archaeal (-A), or eukaryotic (-E) maximum sensitivity mode (-I --max)⁶¹. tRNAscan-SE detection parameters were chosen according to the predicted host of the corresponding viral strain.

Mobile tRNAome tRNA library generation and selection

We generated our mobile tRNAome expression library by synthesizing tRNAscan-SE predicted tRNAs from diverse sources (Supplementary Data 1), driven by a strong bacterial *proK* tRNA promoter and followed by two transcriptional terminators as 10 pmol ssDNA oligonucleotide libraries (10 pmol oPool, from Integrated DNA Technologies, USA). Oligonucleotides were resuspended in 1× TE buffer and then amplified using 5' phosphorylated primers. Amplicons were then blunt-end ligated into pCR4Blunt-TOPO (Invitrogen, Zero Blunt™ TOPO™ PCR Cloning Kit) for 18 hrs at 16 °C and then purified by using the Thermo Scientific GeneJET PCR Purification Kit. We then electroporated 50 ng purified plasmid in five parallel electroporations into 5 × 40 µl freshly made electrocompetent cells of MDS42 and Syn61-3(ev5). Prior to electrotransformation, bacterial cells were made electrocompetent by growing cells after a 1:100 dilution in SOB until mid-log phase (OD=0.3) at 32 °C and then washing cells three times using ice-cold water. Electroporated cultures were allowed to recover overnight at 37 °C and then plated to LBL agar plates containing 50 µg/ml kanamycin in 145×20 mm Petri dishes (Greiner Bio-One). Plates were incubated at 37 °C until colony formation. Approximately 1000–5000 colonies were then washed off from selection plates, and plasmids were extracted by using the Monarch® Plasmid Miniprep Kit (New England Biolabs). The tRNA insert from isolated plasmids was then amplified with primers bearing the standard Nextera Illumina Read 1 and Read 2 primer binding sites, barcoded using the IDT 10 bp UDI indices, and sequenced on an Illumina NextSeq 2000, producing 150 bp paired-end reads. Demultiplexing was

performed with bcl-convert (v3.9.3). Paired-end reads were then trimmed using BBDuk from BBTools (in Geneious Prime[®] 2022.1.1., Biomatters Ltd.), merged, and aligned to their reference sequences by using Geneious assembler, implemented in Geneious Prime[®] 2022.1.1., allowing maximum a single SNV within the tRNA read.

tRNA-Leu_{YGA} library generation and selection

We identified leucine tRNAs that can translate TCR codons as leucine by performing two consecutive screens with plasmid libraries expressing an anticodon loop mutagenized 65,536-member library of *leuU* tRNA variants and a smaller, 13-member tRNA-Leu_{YGA} expression library consisting of bacteriophage derived Leu tRNA variants, both bearing two tRNAs under the control of a *proK* promoter and with anticodons swapped to UGA and CGA. To construct a 65,536-member *leuU* tRNA library, we synthesized an expression construct consisting of a *proK* promoter-leuU_{UGA}-spacer-leuU_{CGA}-*proK* terminator sequence, in which the anticodon loop of both *leuU* tRNAs has been fully randomized, as an oPool library (Supplementary Data 3) (Integrated DNA Technologies, USA). Next, we amplified these *leuU* variants by using Q5 Hot Start High-Fidelity Master mix using 5' phosphorylated primers and then ligated the library into a plasmid backbone containing a high copy-number pUC origin-of-replication and an APH(3')-I aminoglycoside O-phosphotransferase (*aph3Ia*_{29×Leu→TCR}) gene in which all 29 instances of leucine coding codons were replaced with TCR serine codons (synthesized as a gBlock dsDNA fragment by Integrated DNA Technologies, USA). The ligation was performed at a 3:1 insert-to-vector ratio and by using T4 DNA ligase (New England Biolabs, USA) for 16 hours at 16 °C according to the manufacturer's instructions. Finally, the ligation product was purified using the GeneJet PCR purification kit (Thermo Fischer Scientific, USA). We constructed the second, 13-member tRNA-Leu_{YGA} expression library (Supplementary Data 3) consisting of bacteriophage-derived Leu tRNA variants bearing a UGA and CGA anticodon by using the same method as for our *leuU*_{YGA} library. Following library generation, 100 ng from each library was electroporated into freshly made electrocompetent cells of Syn61 3 (ev5) *recA* (ev1) and recovered in SOB at 37 °C for 16 hours, 250 rpm. After recovery, the cells were plated to 2×YT agar plates containing kanamycin at 200 µg/ml concentration, and selection plates were incubated at 37 °C until colony formation. Finally, plasmids from clones were purified using a Monarch plasmid miniprep kit (New England Biolabs, USA) and subjected to whole plasmid sequencing (SNPsaurus, Eugene, Oregon, US).

Virus resistance analysis of Ec_Syn61 3-SL cells

An exponential phase culture (OD₆₀₀ = 0.3) of the corresponding strain was grown in 3 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂ and 75 µg/ml kanamycin at 37 °C with shaking. Cultures were then spun down and resuspended in 1 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂ and infected with a 1:1 mixture of all 12 Syn61 3-lytic phage isolates from this study (Extended Data Table 1b) at an MOI of 0.1. Infected cultures were incubated at 37 °C without shaking for 10 minutes for phage attachment and then washed three times with 1 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂ by pelleting cells at 4000× g for 3 minutes. Infected cells were then diluted into 4 ml SOB supplemented with 10 mM CaCl₂ and MgCl₂ and 75 µg/ml kanamycin and incubated at 37 °C with shaking at 250 rpm. After 24 hours of incubation, 500 µl samples were measured out into a

sterile Eppendorf tube containing 50 μ l chloroform, immediately vortexed, and then placed on ice. Phage infection experiments were performed in three independent replicates. Phage titers were determined by centrifuging chloroformed cultures at 6000 \times g for 3 minutes and then plating 5 μ l of the supernatant directly or its appropriate dilutions mixed with 300 μ l MDS42 cells in LBL 0.7% top agar with 10 mM CaCl₂ and MgCl₂. Following 18 hours of incubation at 37 °C, plaques were counted, and the number of plaques was multiplied by the dilution to calculate the phage titer of the original sample.

Phage enrichment experiments were performed by mixing 50 ml early exponential phase cultures (OD₆₀₀ = 0.2) of bipA-biocontained Ec_Syn61 3-SL carrying pLS1 and pLS2 plasmids with 10 ml environmental sample mix, containing the mixture of Sample 2–13 from our study (Extended Data Table 1b). We maximized phage diversity in the environmental sample mix by using freshly collected and filter-sterilized sewage. Infected Ec_Syn61 3-SL cells with the corresponding plasmid were grown overnight in SOB supplemented with 200 mM bipA, 10 mM CaCl₂ and MgCl₂, and 75 μ g/ml kanamycin at 37 °C with shaking at 250 rpm. On the next day, cells were removed by centrifugation at 4000 \times g for 20 minutes, and the supernatant was filter-sterilized using a 0.45 μ m filter. Next, 5 ml of the sterilized sample was mixed again with 50 ml early exponential phase cultures (OD₆₀₀ = 0.2) of the corresponding strain, incubated for 20 minutes at 37 °C for phage absorption, pelleted by centrifugation at 4000 \times g for 15 minutes, and then resuspended in 50 ml SOB supplemented with 200 mM bipA, 10 mM CaCl₂ and MgCl₂, and 75 μ g/ml kanamycin. Infected cultures were then incubated at 37 °C with shaking at 250 rpm. Cultures were grown overnight and then sterilized by centrifugation at 4000 \times g for 15 minutes and filtered through a 0.45 μ m PVDF Steriflip™ disposable vacuum filter unit (MilliporeSigma). Finally, phage titers were determined by using MDS42 cells as above. Phage enrichment experiments were performed in two independent replicates. The lytic phage titer of the unenriched sample mix was determined by diluting 100 μ l of the input environmental sample mix, containing a mixture of Sample 2–13 (Extended Data Table 1), in SM buffer and 10 μ l samples from each dilution steps were mixed with late-exponential phase MDS42 cells and 4 ml 0.7% top agar, and then poured on top of LBL agar plates. Plates were incubated until plaque formation at 37 °C.

Construction of pLS plasmids

All pLS plasmids listed in Supplementary Data 3 were synthesized as gBlocks by IDT and circularized either by ligation with T4 DNA ligase (New England Biolabs, USA), or, in the case of pSC101 and RK2 plasmid-derived variants, by isothermal assembly using the HiFi DNA Assembly Master Mix (New England Biolabs, USA). Following assembly, purified plasmid assemblies were electroporated into Ec_Syn61 3-SL cells carrying pLS1. pLS1 and pLS2 were designed to express two distinct combinations of the previously identified phage tRNA-Leu_{YGA} tRNAs in antiparallel orientation to avoid repeat-mediated instability⁶², driven by the strong bacterial *proK* and SLP2018–2-101 promoters, together with a high copy-number pUC origin-of-replication, the *aph3Ia*_{29 \times Leu \rightarrow TCR} and *aminoglycoside-(3)-N-acetyltransferase*_{18 \times Leu \rightarrow TCR} marker genes. Transformants carrying either pLS1 or pLS2 were identified by transforming assemblies into Syn61 3(ev5) *recA* (ev1) and selecting for kanamycin resistance. Finally, plasmids from antibiotic-resistant clones were purified

using a Monarch plasmid miniprep kit (New England Biolabs, USA) and subjected to whole plasmid sequencing (SNPsaurus, Eugene, Oregon, US).

Escape rate analysis of viral Leu-tRNA_{YGA}s and pLS plasmids

We analyzed the ability of pLS plasmids to function outside Ec_Syn61³-SL cells by transforming extracted plasmids into *E. coli* K-12 MG1655. Plasmids were purified from biocontained Ec_Syn61³-SL cells, carrying either pLS1 or pLS2 to express tRNA-Leu_{YGA}, or pLS1 together with pLS3–5, by using the PureLink™ Fast Low-Endotoxin Midi Plasmid Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Next, we electroporated 1 µg from each plasmid prep into freshly made electrocompetent cells of *E. coli* K-12 MG1655. Cells were made electrocompetent by diluting an overnight SOB culture of MG1655 1:100 into 500 ml SOB in a 2000 ml flask and growing cells aerobically at 32 °C with shaking at 250 rpm. At OD₆₀₀ = 0.3, cells were cooled on ice and then pelleted by centrifugation and resuspended in 10% glycerol-in-water. Cells were washed 4-times with 10% glycerol-in-water and then resuspended in 400 µl 20% glycerol-in-water. 1000 ng from each plasmid sample was then mixed with 80 µl electrocompetent cells and electroporated by using standard settings in two 1-mm electroporation cuvettes by using standard electroporator settings (1.8 kV, 200 Ohm, 25 µF). Electroporations were performed in three independent replicates. Electroporated cells were then resuspended in 1 ml SOB, and the culture was allowed to recover overnight at 37 °C with shaking at 250 rpm. Finally, 500 µl from each recovery culture was plated to LBL agar plates containing antibiotics corresponding to the given pLS plasmid's resistance marker (15 µg/ml gentamycin plus 50 µg/ml kanamycin in the case of pLS1 and 2; 100 µg/ml carbenicillin for pLS4, 30 µg/ml chloramphenicol for pLS3 and pLS6, and 20 µg/ml gentamycin for pLS5) in 145×20 mm Petri dishes (Greiner Bio-One). Plates were incubated at 37 °C for seven days and inspected for growth. Electroporation efficiency measurements were performed by electroporating a plasmid carrying a pUC origin-of-replication and kanamycin resistance (pUC-Kan^R) into MG1655 electrocompetent cells under identical conditions.

Cloning of REP12 tRNA operon

We analyzed the incorporated amino acid in Syn61³ cells bearing the tRNA operon and its native promoter from the REP12 phage by subcloning the genomic tRNA operon into a low-copy plasmid containing an RK2 origin-of-replication and a chloramphenicol-acetyltransferase marker, both recoded to contain no TCR or TAG codons. The genomic tRNA operon of REP12 was PCR amplified using the Q5 Hot Start Master Mix (New England Biolabs, USA) from extracted phage gDNA and purified using the GeneJet PCR purification kit (Thermo Fischer Scientific, USA). Next, 100 ng of the amplified tRNA operon was assembled into the linearized pRK2-*cat* backbone using the HiFi DNA Assembly Master Mix (New England Biolabs, USA). After incubation for 60 mins at 50 °C, the assembly was purified using the DNA Concentrator & Clean kit (Zymo Research, USA) and transformed into Syn61³(ev5) *recA* (ev1) cells expressing an MSKGPVKVPGAGVPGxGVPGVKGKGGT- elastin peptide fused to sfGFP with a terminal 6×His tag (in which x denotes the analyzed codon, TCA or TCG) on a plasmid containing a kanamycin resistance gene and a pUC origin of replication⁶³. Following an

overnight recovery at 32 °C, cells were plated to 2×YT agar plates containing kanamycin and chloramphenicol. Finally, plasmid sequences in outgrowing colonies were validated by whole-plasmid sequencing. Elastin_{16TCA}-sfGFP-6×HIS expression measurements have been performed as described below.

Elastin_{16TCA}-sfGFP-6×HIS expression measurements

We assayed the amino acid identity of the serine tRNA_{UGA} tRNAs of MZ501075 and REP12 (see Supplementary Data 3 for sequence information) by coexpressing selected tRNAs and a constitutively expressed MSKGPVKVPGAGVPGxGVPGVVGKGGGT- elastin peptide fused to sfGFP with a terminal 6×His tag (in which **x** denotes the analyzed codon, TCA or TCG)⁶³ on a plasmid containing a kanamycin resistance gene and a pUC origin-of-replication in Syn61 3(ev5). Similarly, the pRK2-REP12 plasmid carrying the REP12 phage tRNA operon under the control of its native promoter was coexpressed with the same pUC plasmid carrying no tRNA genes. As a control, we utilized the same elastin-sfGFP-6×HIS expression construct in which position **x** has been replaced with an alanine GCA codon. For fluorescence and MS/MS measurements, we diluted cultures 1:100 from overnight starters into 50 ml 2×YT in 300 ml shake flasks containing the corresponding antibiotics and cultivated for 48 hours at 37 °C, 200 rpm aerobically. We then determined sfGFP expression levels in samples by pelleting and washing 1 ml of the culture with PBS and resuspending cell pellets in 110 µL BugBuster Protein Extraction Reagent (MilliporeSigma). Reactions were incubated for 5 minutes and then spun down at 13,000× rpm for 10 minutes. The fluorescence of the BugBuster-treated supernatants and the OD₆₀₀ of the original culture was measured using the Synergy H1 Hybrid Reader (BioTek) plate reader using the bottom mode analysis with an excitation at 480 nm and emission measurement at 515 nm, with the gain set to 50. Fluorescence values were normalized based on OD₆₀₀ data. The remaining 49 mL culture was spun down, and the cell pellet was resuspended in 2 ml BugBuster Protein Extraction Reagent (MilliporeSigma) and incubated at room temperature for 5 minutes. The lysed cell mixture was spun down at 13,000× rpm for 10 minutes, and the supernatant was mixed in a 1:1 ratio with HIS-Binding/Wash Buffer (G-Biosciences, USA) and 50 µl HisTag Dynabeads (Thermo Fischer Scientific, USA). Following an incubation period of 5 minutes, the beads were separated on a magnetic rack and washed with 300 µl HIS-Binding/Wash Buffer and PBS (Phosphate Buffered Saline) three times. After the last wash step, the bead pellets containing the bound elastin-sfGFP-6×HIS protein samples were frozen at -80 °C until MS/MS sample preparation. Protein production experiments were performed in three independent replicates.

Tandem liquid chromatography and mass spectrometry (LC/MS-MS) analysis of tryptic elastin-sfGFP-6×HIS

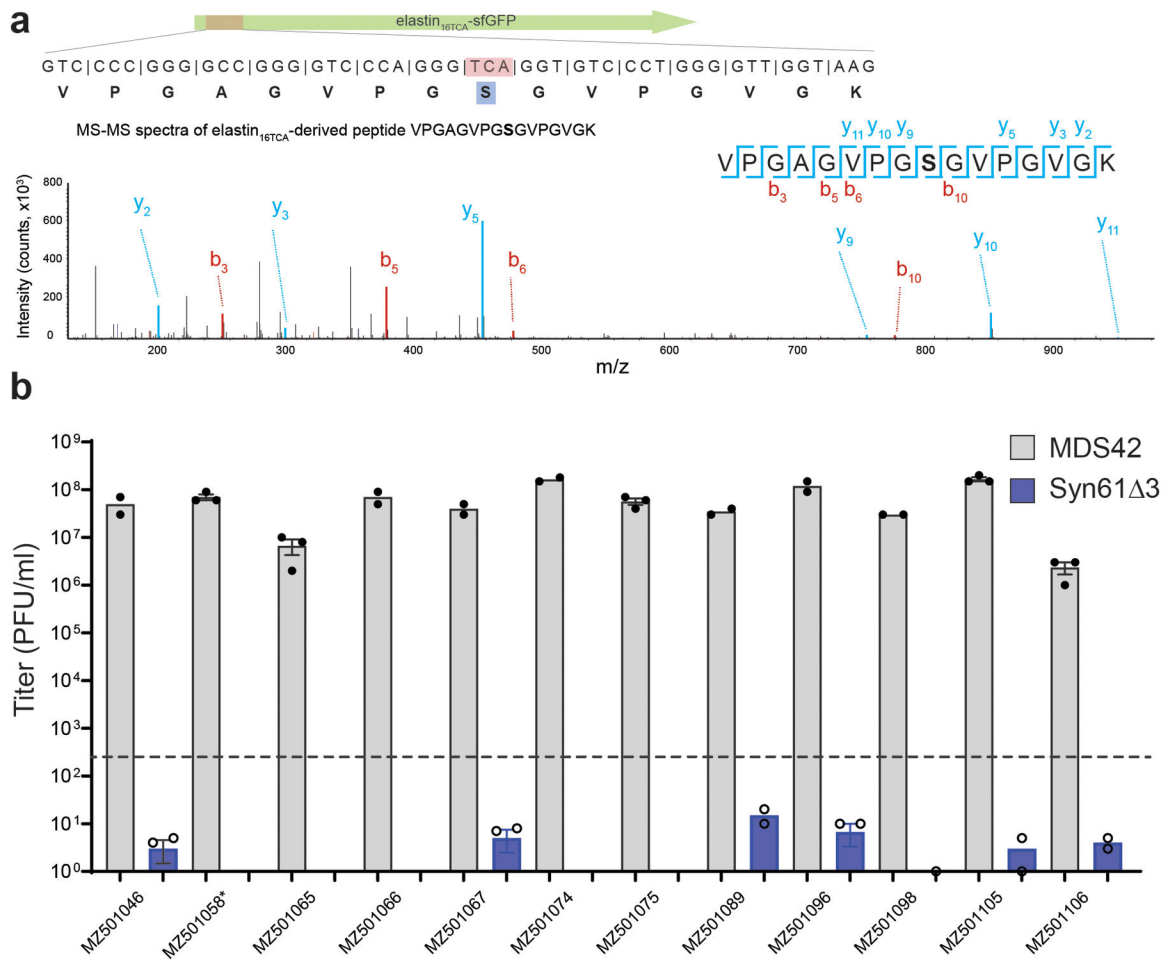
Samples from elastin-sfGFP-6×HIS expression experiments were digested directly on HisTag Dynabeads according to the FASP digest procedure⁶⁴. In brief, samples were washed with 50 mM TEAB (triethylammonium bicarbonate buffer) and then rehydrated with 50 mM TEAB-trypsin solution, followed by a three-hour digest at 50 °C. Digested peptides were then separated from HisTag Dynabeads and concentrated by spinning and drying samples at 3,000× rpm using a SpeedVac concentrator. Samples were then solubilized in 0.1% formic acid-in-water for subsequent analysis by tandem mass spectrometry. LC-MS/MS analysis of

containing 10 mM CaCl₂, MgCl₂, and 75 µg/ml kanamycin were spun down at room temperature and resuspended in 1.1 ml SOB containing 10 mM CaCl₂, MgCl₂, and 75 µg/ml kanamycin. 100 µl of this uninfected sample was immediately frozen in liquid N₂ and stored at -80 °C until proteome analysis. Next, 1000 µl of this cell suspension was mixed with 10 ml prewarmed REP12 phage stock (*i.e.*, ~5×10¹¹ PFU to achieve a MOI of ~12) in SOB containing 10 mM CaCl₂, MgCl₂, and 75 µg/ml kanamycin, and then incubated at 37 °C for 10 minutes without shaking for phage absorption. Following phage attachment, samples were spun down, the supernatant containing unabsorbed phages was removed, and the cell pellet was resuspended in 5 ml SOB containing 10 mM CaCl₂ and MgCl₂. Samples were then incubated at 37 °C, 250 rpm. After 20- and 40-minutes post-infection, 1 ml cell suspensions were spun down, and cell pellets were frozen in liquid N₂ and stored at -80 °C until total protein extraction. Samples from control and phage-infected Syn61 3 cells were then digested by using the FASP digest procedure⁶⁴. In brief, samples were washed with 50 mM TEAB buffer on a 10 kDa cutoff filter (Pall Corp, CA) and then rehydrated with 50 mM TEAB-trypsin solution, followed by a three-hour digest at 37 °C. Digested peptides were then extracted and separated into 10 fractions by using the Pierce™ High pH Reversed-Phase Peptide Fractionation Kit according to the manufacturer's protocol (Thermo Fisher Scientific, USA). Following fractionation, peptides were concentrated and dried by spinning samples at 3.000× rpm using a SpeedVac concentrator. Samples were then solubilized in 0.1% formic acid-in-water for subsequent analysis by tandem mass spectrometry. LC-MS/MS analysis of digested samples was performed on a Lumos Tribrid Orbitrap Mass Spectrometer equipped with an Ultimate 3000 nano-HPLC (both from Thermo Fisher Scientific, USA). Peptides were separated on a 150 µm inner diameter microcapillary trapping column packed first with 2 cm of C18 Repronil resin (5 µm, 100 Å, from Dr. Maisch GmbH, Germany) followed by a 50 cm analytical column (PharmaFluidics, Belgium). Separation was achieved by applying a gradient from 5% to 27% acetonitrile in 0.1% formic acid over 90 mins at 200 nl/min. Electrospray ionization was performed by applying a voltage of 2 kV using a custom electrode junction at the end of the microcapillary column and sprayed from metal tips (PepSep, Denmark). The mass spectrometry survey scan was performed in the Orbitrap in the range of 400–1,800 m/z at a resolution of 6×10⁴, followed by the selection of the twenty most intense ions for fragmentation using Collision Induced Dissociation in the second MS step (CID-MS2 fragmentation) in the Ion trap using a precursor isolation width window of 2 m/z, AGC (automatic gain control) setting of 10,000 and a maximum ion accumulation of 100 ms. Singly charged ion species were excluded from CID fragmentation. The normalized collision energy was set to 35 V and an activation time of 10 ms. Ions in a 10-ppm m/z window around ions selected for MS-MS were excluded from further selection for fragmentation for 60 seconds.

The raw data was analyzed using Proteome Discoverer 2.4 (Thermo Fisher Scientific, USA). Assignment of MS/MS spectra was performed using the Sequest HT algorithm by searching the data against a protein sequence database, including all protein entries from *E. coli* K-12 MG1655, all protein sequences of the corresponding REP12 bacteriophage and the Aph3Ia APH(3')-I aminoglycoside O-phosphotransferase, as well as other known contaminants such as human keratins and common lab contaminants. Quantitative analysis between samples was performed by LFQ (label free quantitation) between different samples.

Sequest HT searches were performed using a 10-ppm precursor ion tolerance and requiring each peptides N-/C termini to adhere with trypsin protease specificity while allowing up to two missed cleavages. Methionine oxidation (+15.99492 Da), deamidation (+0.98402 Da) of asparagine and glutamine amino acids, phosphorylation at serine, threonine, and tyrosine amino acids (+79.96633 Da) and N-terminus acetylation (+42.01057 Da) was set as variable modifications. Special modification of serine to leucine amino acid exchange (+26.052036 Da) on all serine amino acid positions was used as variable modification. All cysteines were set to permanent no modification due to no alkylation procedure. An overall false discovery rate of 1% on both protein and peptide levels was achieved by performing target-decoy database search using Percolator⁶⁵.

Extended Data

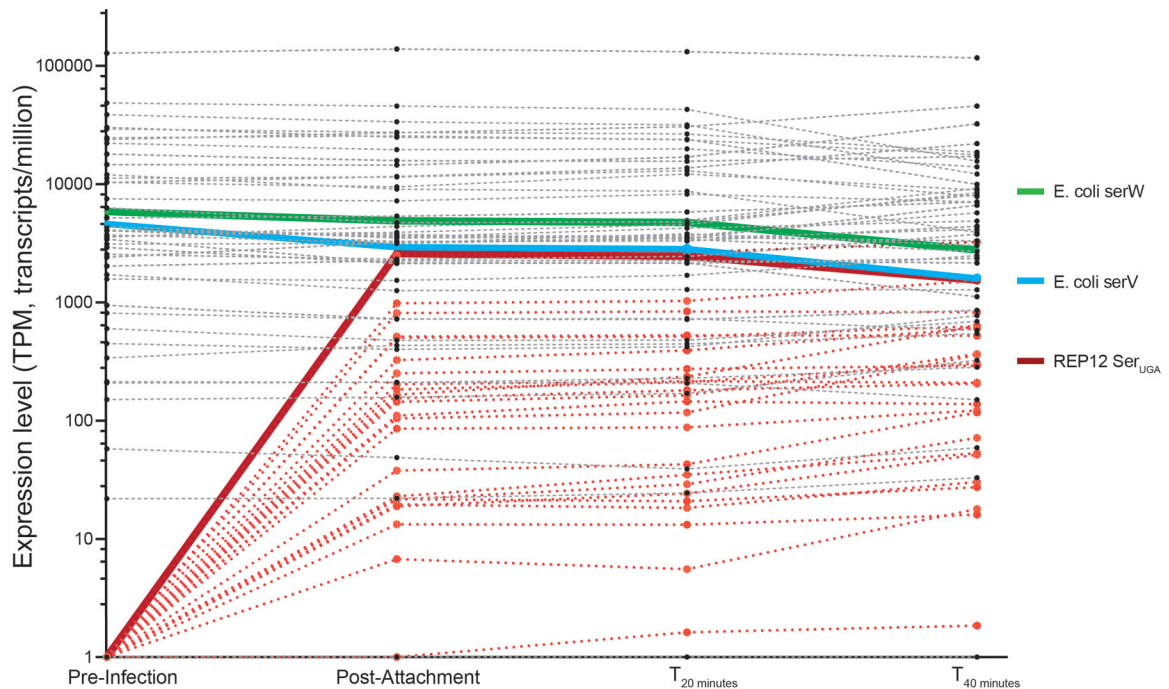


Extended Data Figure 1. Viral serine tRNAs decode TCA codons as serine, but Syn61 Δ 3 obstructs the replication of viruses containing genomic tRNA-Ser_{UGA}.

a) Viral TCR suppressor tRNAs decode TCA codons as serine. The amino acid identity of the translated TCR codon within elastin₁₆ TCA-sfGFP-His6 was confirmed by tandem mass spectrometry from Syn61 Δ 3 expressing the tRNA-Ser_{UGA} of *Escherichia* phage

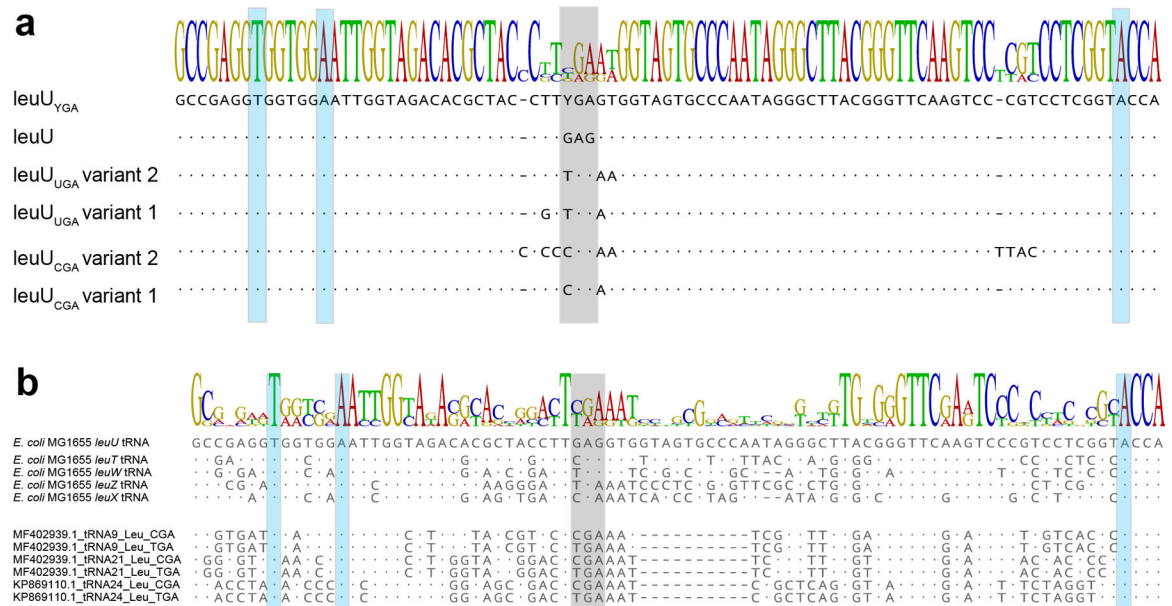
IrisVonRoten (GenBank ID [MZ501075](#))²⁴. The figure shows the amino acid sequence and MS/MS spectrum of the analyzed elastin₁₆ TCA peptide. MS/MS data was collected once.

b) Syn61-3 obstructs the replication of viruses containing genomic tRNA-Ser_{UGA}. Figure shows the titer of twelve tRNA gene-containing coliphages²⁴, after 24 hours of growth on MDS42 and Syn61-3. All analyzed bacteriophages, except MZ501058, contain a genomic tRNA-Ser_{UGA} tRNA that provides TCR suppressor activity based on our screen (Figure 1b, Supplementary Data 1). Early exponential phase cultures of MDS42 and Syn61-3 were infected at an MOI of 0.001 with the corresponding phages, and free phage titers were determined after 24 hours of incubation. Measurements were performed in n=3 independent experiments (*i.e.*, MDS42 + MZ501058, MZ501065, MZ501075, MZ501105, MZ501106; Syn61-3 + MZ501046, MZ501067, MZ501066, MZ501096, MZ501074, MZ501098) or in n=2 independent experiments (*i.e.*, MDS42 + MZ501046, MZ501066, MZ501067, MZ501074, MZ501089, MZ501096, MZ501098; Syn61-3 + MZ501058, MZ501065, MZ501075, MZ501089, MZ501105, MZ501106); dashed line represents input phage titer without bacterial cells (*i.e.*, a titer of 420 PFU/ml); dots represent data from independent experiments; bar graphs represent the mean; error bars represent the SEM based on n=3 independent experiments.



Extended Data Figure 2. Viruses overcome genetic-code-based resistance by rapidly complementing the cellular tRNA pool with virus-encoded tRNAs.

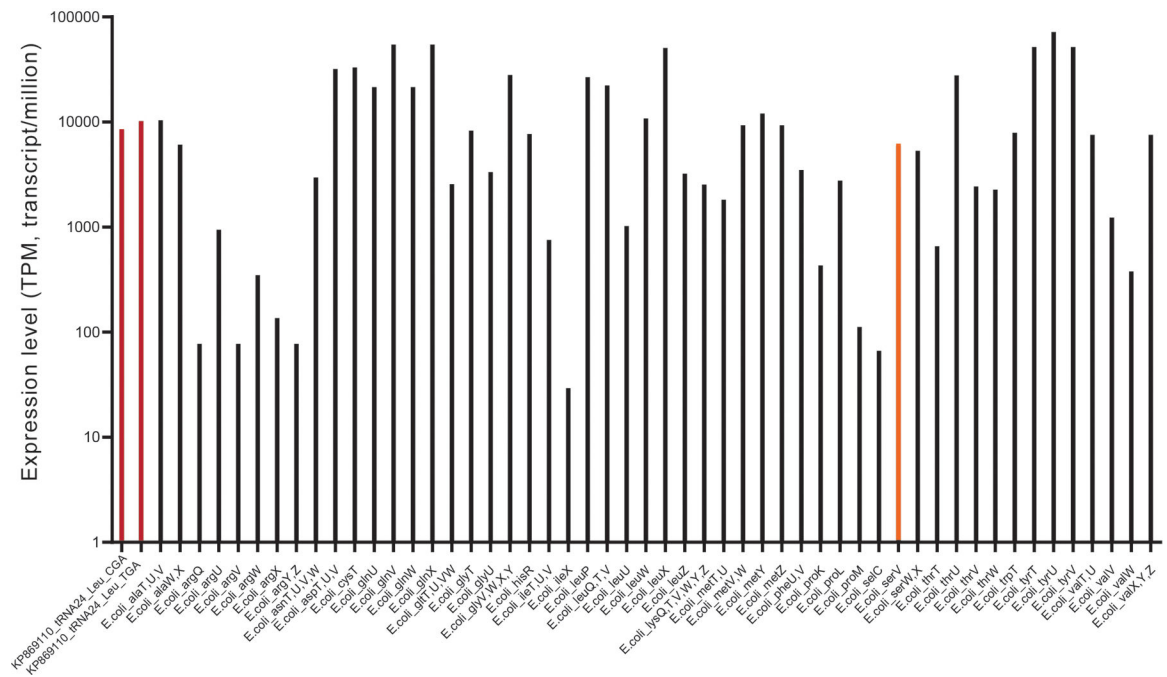
The time-course kinetics of host and viral tRNA expression in Syn61-3 cells following REP12 phage infection was quantified using tRNAseq (Methods). The endogenous *serV* and *serW* tRNAs of the host Syn61-3 are highlighted in green and blue, respectively, while the tRNA-Ser_{UGA} of the REP12 virus is highlighted in red. REP12 viral tRNAs are shown in light red; endogenous tRNAs of Syn61-3 are shown in gray. Data represent mean TPM (transcript/million). Source data is available within this paper.



Extended Data Figure 5. Multiple sequence alignment of *leuUYGA* and phage-derived tRNA-*LeuYGA* variants.

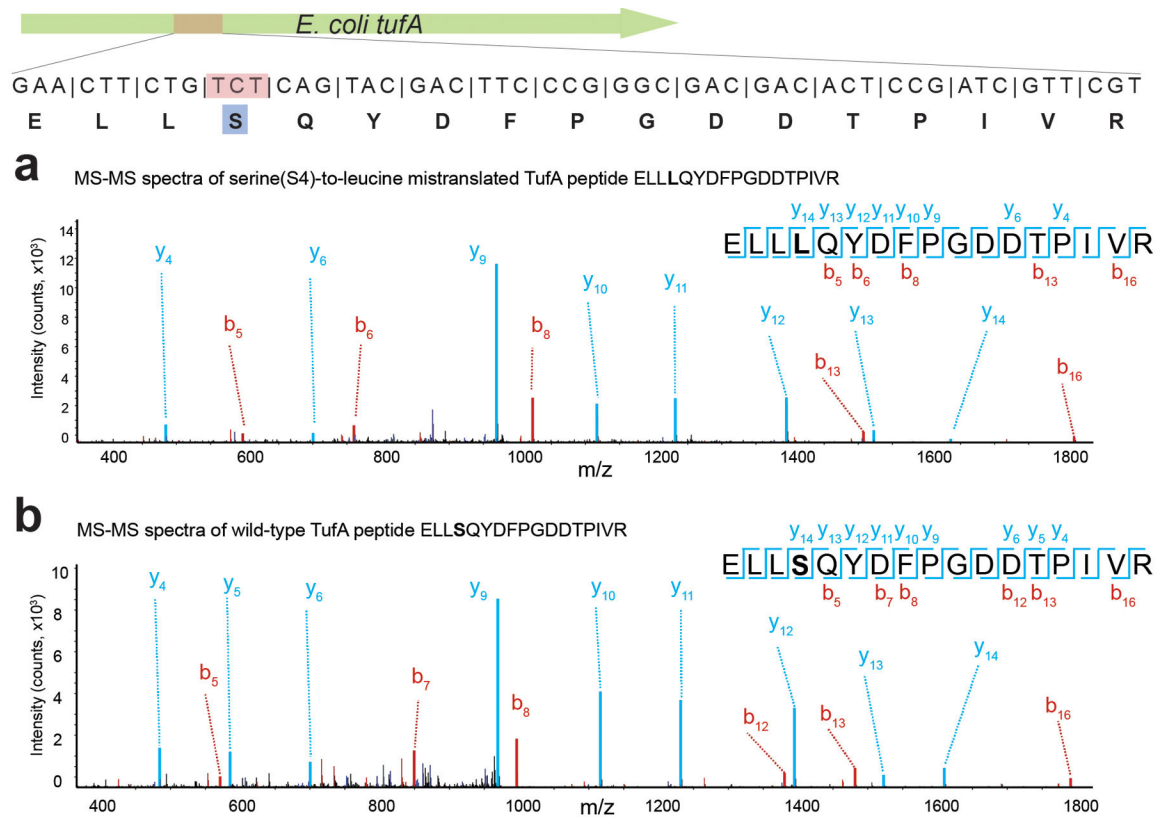
a) Multiple sequence alignment of *leuUYGA* variants selected in aminoglycoside O-phosphotransferase expression screen, compared to *E. coli leuU* and the YGA anticodon-swapped *E. coli leuU* tRNA variant. Grey shading indicates the anticodon region, and the host's *LeuS* leucine-tRNA-ligase identity elements³¹ are shown in blue. Sequence information of the *leuUYGA* variants is available in Supplementary Data 3.

b) Multiple sequence alignment of phage-derived tRNA-*LeuYGA* variants selected in the *aph3Ia*₂₉×*Leu*→*TCR* aminoglycoside O-phosphotransferase expression screen, compared to endogenous *E. coli* leucine tRNAs. Grey shading indicates the anticodon region, while the host's *LeuS* leucine-tRNA-ligase identity elements³¹ are shown in blue. Sequence information of the phage-derived tRNA-*LeuYGA* variants is available in Supplementary Data 3.



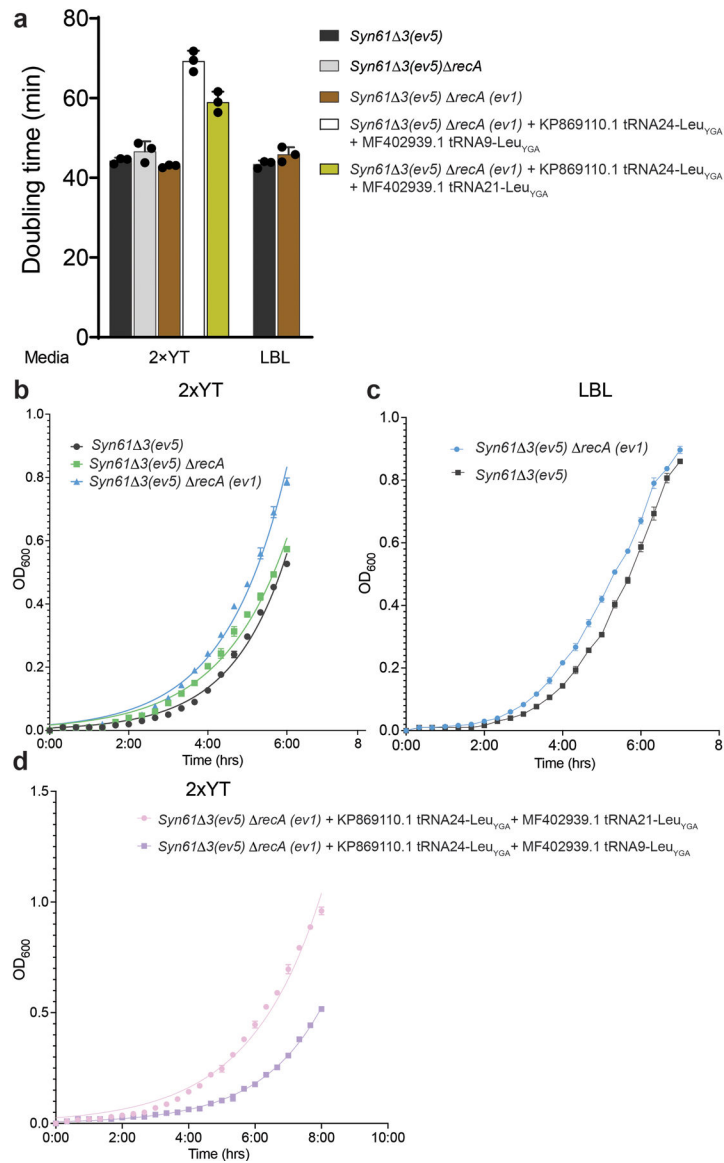
Extended Data Figure 6. tRNAseq-based quantification of viral Leu-tRNA_{UGA} and Leu-tRNA_{CGA} in the tRNAome of Ec_Syn61 3-SL.

tRNA levels of Ec_Syn61 3-SL were quantified using tRNAseq (Methods). Viral Leu-tRNA_{UGA} and Leu-tRNA_{CGA} are highlighted in red, and the host's endogenous *E. coli serV* tRNA is highlighted in orange. tRNAseq data was collected once. Data represent TPM (transcript/million). Source data is available within this paper.



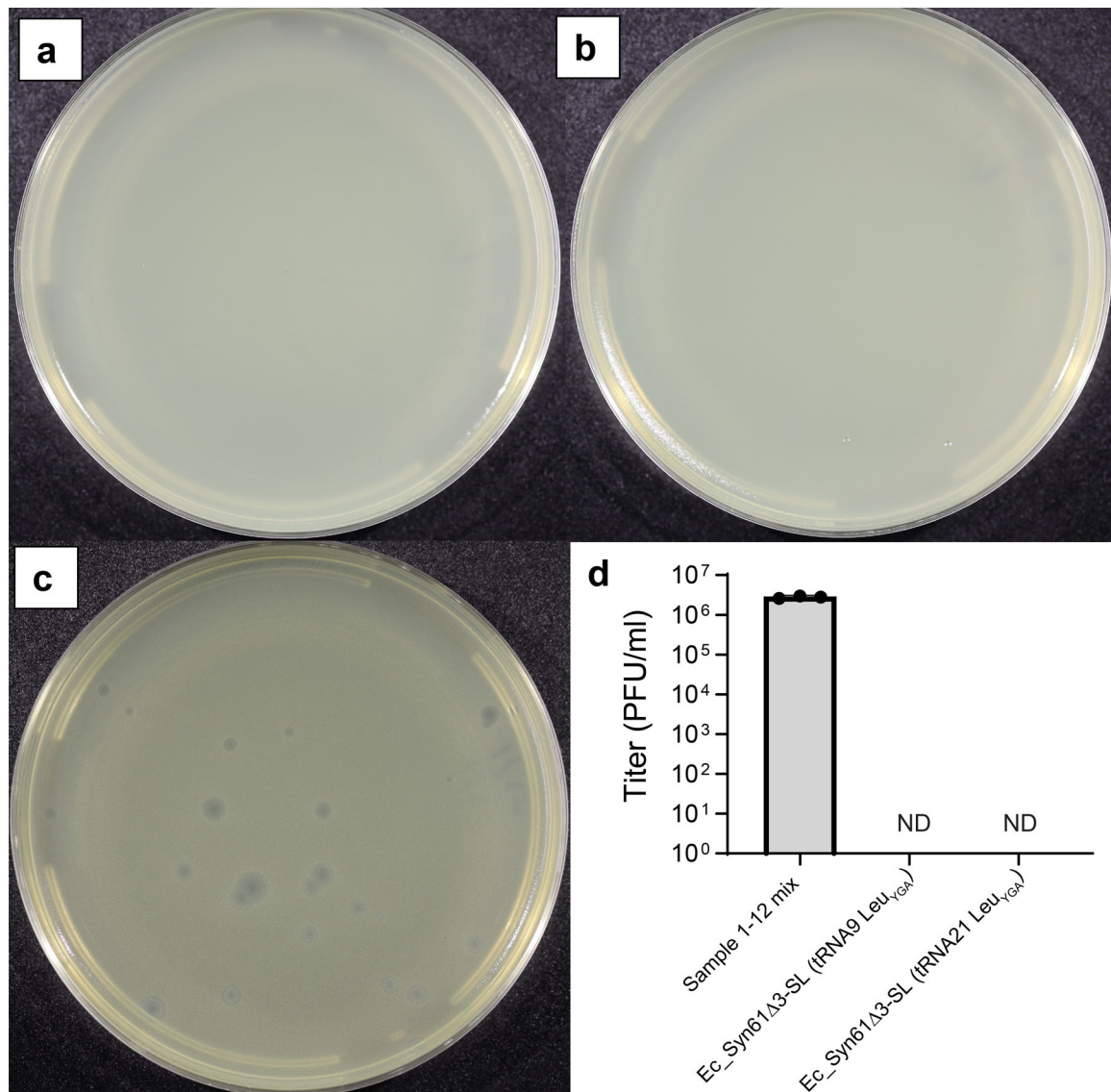
Extended Data Figure 7. Serine-to-leucine mistranslation of TCT codons in *Ec_Syn61* 3-SL cells.

a) MS/MS spectrum of the serine-to-leucine mistranslated TufA peptide. **b)** MS/MS spectrum of the wild-type TufA peptide. The figure shows the amino acid sequence and MS/MS spectrum of the *Ec_Syn61* 3-SL-expressed TufA protein fragment, together with its genomic sequence, in which the serine-coding TCT codon (as shown in panel **b**) is partially mistranslated as leucine (as shown in panel **a**). The experiment was performed by analyzing the total proteome of *Ec_Syn61* 3-SL cells, expressing Leu9-tRNA_{YGA} from *Escherichia* phage OSYSP (GenBank ID [MF402939.1](#)) by tandem mass spectrometry (Methods). MS/MS data was collected once.



Extended Data Figure 8. Doubling time and growth curves of *Syn61* 3(*ev5*), *Syn61* 3(*ev5*) *recA*, *Syn61* 3(*ev5*) *recA*(*ev1*), and *Ec_Syn61* 3-SL.

a) Doubling times of *Syn61* 3(*ev5*), *Syn61* 3(*ev5*) *recA*, *Syn61* 3(*ev5*) *recA*(*ev1*), and *Ec_Syn61* 3-SL, calculated based on growth curves (shown in panels **b**, **c**, **d**) in rich bacterial media under standard laboratory conditions. **b)** Growth curves of *Syn61* 3(*ev5*), *Syn61* 3(*ev5*) *recA*, and *Syn61* 3(*ev5*) *recA*(*ev1*) in LBL broth. **c)** Growth curves of *Syn61* 3(*ev5*) and *Syn61* 3(*ev5*) *recA*(*ev1*) in 2xYT broth. **d)** Growth curves of *Ec_Syn61* 3-SL in 2xYT broth containing 50 μg/ml kanamycin. Three independent cultures were grown aerobically in vented shake flasks at 37 °C, and OD₆₀₀ measurements were taken during exponential growth (Methods). Data curves and bars represent the mean. Error bars show standard deviation based on n=3 independent experiments.



Extended Data Figure 9. Ec_Syn61 3-SL resists viruses in environmental samples.

Phage enrichment experiment using Ec_Syn61 3-SL, expressing KP869110.1 tRNA²⁴ Leu_{YGA} and MF402939.1 tRNA⁹ Leu_{YGA}, as host. (b) Phage enrichment experiment using Ec_Syn61 3-SL, expressing KP869110.1 tRNA²⁴ Leu_{YGA} and MF402939.1 tRNA²¹ Leu_{YGA}, as host. Phage enrichment experiments were performed by mixing early exponential cultures of Ec_Syn61 3-SL with 10 ml environmental sample mix containing the mixture of Sample 2–13 from our study (Extended Data Table 1a). After two enrichment cycles (Methods, Supplementary Note), filter-sterilized culture supernatants were mixed with phage-susceptible *E. coli* MDS42 cells in top agar and plated on LBL agar plates to determine viral titer. Enrichment experiments were performed in n=2 independent replicates with the same result. (c) Lytic *E. coli* MDS42 phage plaques after 10³-fold dilution of the environmental sample mix. (d) Lytic phage titer of the environmental sample mix, before and after enrichment on Ec_Syn61 3-SL. Dots represent the viral titer of the unenriched sample based on three independent experiments, measured on *E. coli* MDS42 cells. ND represents

no plaques detected. Bar represents the mean. Error bar shows standard deviation based on n=3 independent experiments.

Extended Data Table 1.
Isolation of lytic viruses infecting Syn61 3.

a) Environmental samples analyzed in this study for the presence of lytic viruses of Syn61 3. Samples containing lytic phages of Syn61 3 are highlighted in gray.
b) Genome size and taxonomy of lytic viruses infecting Syn61 3. Annotated genome sequences of all REP phage isolates are available in the Supplementary Data of this paper. REP is an abbreviation of **Recorded *E. coli* Phage**. The annotated genomes of REP phages have been deposited to NCBI GenBank under Accession numbers [OQ174500](#), [OQ174501](#), [OQ174502](#), [OQ174503](#), [OQ174504](#), [OQ174505](#), [OQ174506](#), [OQ174507](#), [OQ174508](#), [OQ174509](#), [OQ174510](#), and [OQ174511](#).

a			
Sample ID	Sample description	Sample treatment	Sample source location
1	Wastewater (primary effluent)	Chloroform treated	Massachusetts, USA
2	Wastewater (primary effluent)	0.22 µm filtered	Massachusetts, USA
3	River water	0.22 µm filtered	Massachusetts, USA
4	Soil from horse and alpaca enclosure	0.22 µm filtered	Massachusetts, USA
5	Water from porta-potty overflow	0.22 µm filtered	Massachusetts, USA
6	Irrigation water near wild rat nest	0.22 µm filtered	Massachusetts, USA
7	Soil from pasture-raised chicken enclosure	0.22 µm filtered	Massachusetts, USA
8	Soil from egg-laying chicken shed	0.22 µm filtered	Massachusetts, USA
9	Fresh pig feces	0.22 µm filtered	Massachusetts, USA
10	Mixed fresh horse and cow feces	0.22 µm filtered	Massachusetts, USA
11	Farm compost pile	0.22 µm filtered	Massachusetts, USA
12	Farm compost pile	0.22 µm filtered	Massachusetts, USA
13	Water from pig and goose water trough	0.22 µm filtered	Massachusetts, USA

b		
Isolate	Genome size (kb)	Taxonomic classification
REP1	167.4	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Tevenvirinae; Tequatrovirus
REP2	167.9	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Tevenvirinae; Tequatrovirus
REP3	167.9	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Tevenvirinae; Tequatrovirus
REP4	166.8	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Tevenvirinae; Tequatrovirus
REP5	88.9	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Ounavirinae; Felixounavirus
REP6	87.2	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Ounavirinae; Felixounavirus
REP7	88.9	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Ounavirinae; Felixounavirus
REP8	85.8	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Ounavirinae; Felixounavirus

b		
Isolate	Genome size (kb)	Taxonomic classification
REP9	85.8	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Oonavirinae; Felixounavirus
REP10	85.7	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Oonavirinae; Felixounavirus
REP11	85.7	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Oonavirinae; Felixounavirus
REP12	85.8	Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Myoviridae; Oonavirinae; Felixounavirus

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability Statement

Raw data from whole-genome sequencing, transcriptome, and tRNA sequencing experiments have been deposited to Sequence Read Archive (SRA) under BioProject ID PRJNA856259. tRNA data and the generated sequences in this study are included in the Supplementary Data files. Mass spectra and proteome measurements have been deposited to MassIVE (MSV000089854; doi:10.25345/C5FF3M41W)⁶⁶. The Syn61 3(ev5) recA (ev1) strain is available from Addgene (Bacterial strain #189857). We cannot deposit Ec_Syn61 3-SL and Ec_Syn61 3 adk.d6 at Addgene due to the incompatibility of Addgene's methods of strain distribution and growth media requirements, but all materials used in this study are freely available from the corresponding authors for academic research use upon request. The PHROG HMM database is available at <https://phrogs.lmge.uca.fr/> and from reference⁵³. The assembled annotated genome of *Escherichia coli* Syn61 substr. Syn61 3(ev5) is available in Supplementary Data 4, and the annotated genome of REP phages have been deposited to NCBI GenBank under accession numbers [OQ174500](#), [OQ174501](#), [OQ174502](#), [OQ174503](#), [OQ174504](#), [OQ174505](#), [OQ174506](#), [OQ174507](#), [OQ174508](#), [OQ174509](#), [OQ174510](#), and [OQ174511](#). Source data is provided with this paper.

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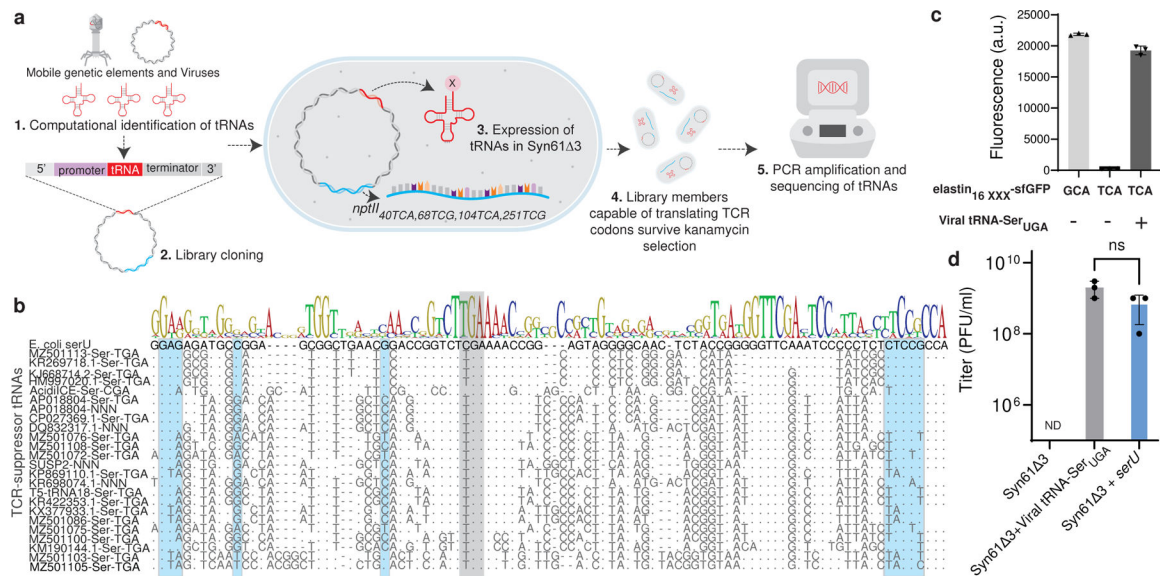


Figure 1. Discovery of mobile TCR codon translating tRNAs in *E. coli* Syn61 3.

(a). We screened the mobile tRNAome for tRNAs that can simultaneously translate TCA and TCG (together TCR) codons by computationally identifying tRNA genes in mobile genetic elements (1.) and then synthesizing select candidates as an oligonucleotide library and cloning these variants into a plasmid vector carrying a *nptII*_{40TCA,68TCG,104TCA,251TCG} marker (conferring kanamycin resistance) (2.). Following the transformation of this library into Syn61 3 (3.), in which the deletion of *serU* (encoding tRNA-Ser_{CGA}) and *serT* (encoding tRNA-Ser_{UGA}) makes TCG and TCA codons unreadable, only variants carrying functional TCR suppressor tRNAs survive kanamycin selection (4.). Finally, high-throughput sequencing of the tRNA inserts from kanamycin-resistant clones identified suppressor tRNAs (5.).

(b). Multiple sequence alignment of mobile TCR codon translating tRNAs. Grey shading indicates the anticodon region, while the host's serine-tRNA-ligase identity elements are shown in blue.

(c). Viral serine tRNA_{UGA} translates the TCA codon. Syn61 3 expressing elastin₁₆ GCA (alanine)-sfGFP-His6 served as wild-type expression control, and the elastin₁₆ TCR-sfGFP-His6 expression was compared with and without the coexpression of the tRNA-Ser_{UGA} of *Escherichia* phage IrisVonRoten²⁴. xxx marks the analyzed codon, TCA or GCA. Bar graph represents the mean; error bars represent SD based on n=3 independent experiments; a.u. denotes arbitrary fluorescence units.

(d). The expression of viral TCR suppressor tRNAs and *serU* (tRNA-Ser_{CGA}) restores the replication of T6 bacteriophage in Syn61 3. Dots represent data from n=3 independent experiments, error bars represent SD, and the bar graph represents the mean. ND represents below the detection limit (*i.e.*, <10³ PFU/ml); ns indicates lack of significance (*p* = 0.116) based on unpaired two-sided Student's *t*-test.

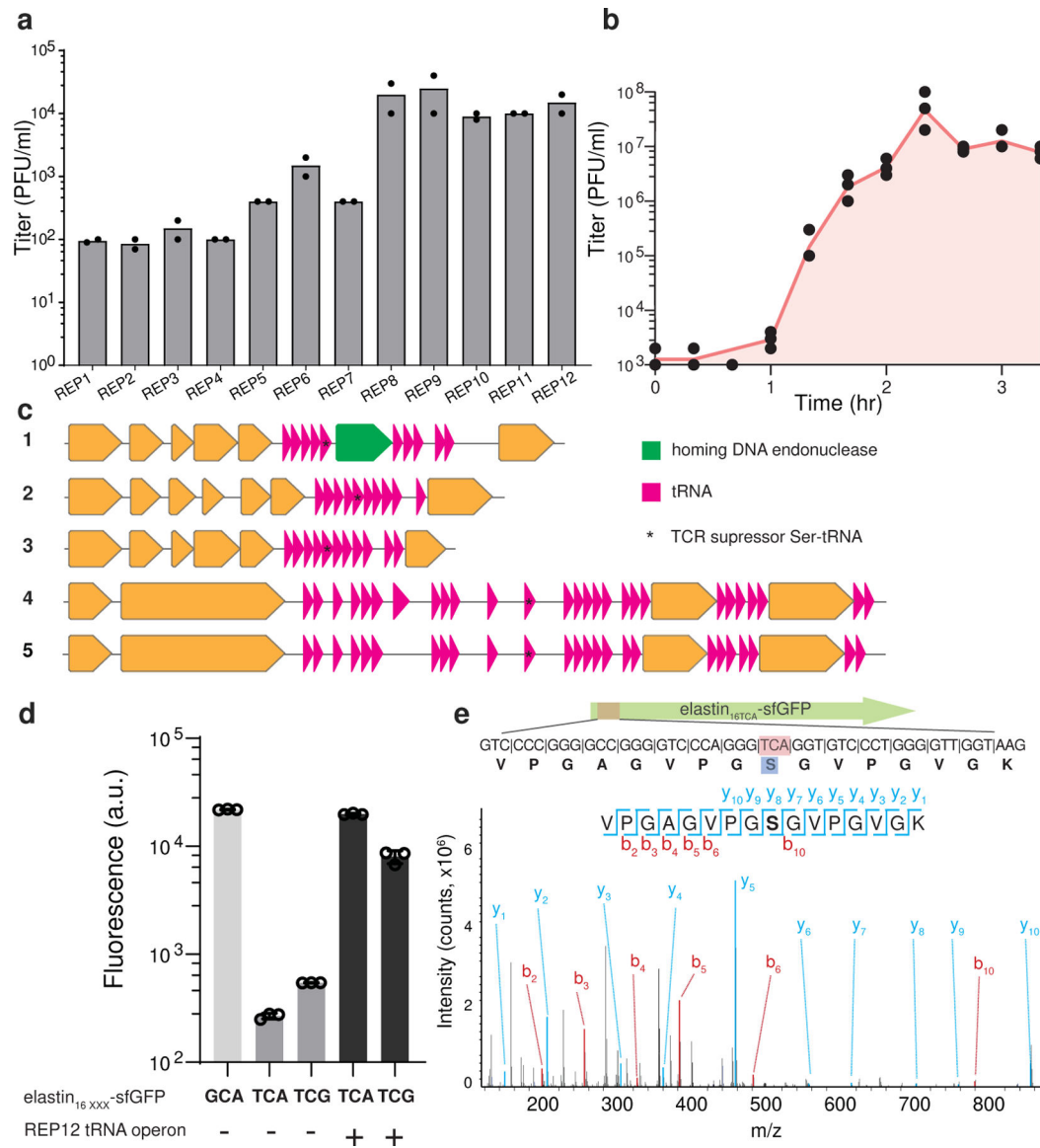


Figure 2. Lytic phages of Syn61-3.

(a). Titer of Syn61-3 phage isolates after replication on Syn61-3. Dots represent data from n=2 independent experiments; bar graphs represent the mean.

(b). Single-step growth curve of REP12 lytic Syn61-3 phage. Single-step growth was performed in n=3 independent experiments, red line represents the mean, and dots represent the total viral titer.

(c). Genomic maps of tRNA operons in lytic Syn61-3 phages. Magenta arrows represent predicted tRNA genes; * denotes tRNA genes identified in our earlier TCR codon suppressor screen (Figure 1b); green arrow represents homing endonuclease gene, while orange arrows represent protein-coding genes. Phage operon numbers correspond to the following REP phages: 1.=REP1; 2.=REP2; 3.=REP4; 4.=REP6; 5.=REP12.

(d). Viral tRNA operon-expressed tRNAs translate TCR codons. Syn61-3 expressing elastin_{16GCA}(alanine)-sfGFP-His6 served as wild-type expression control, and the

elastin₁₆ TCR-sfGFP-His6 expression was compared with and without the coexpression of the REP12 viral tRNA operon. xxx marks the analyzed codon, TCA, TCG, or GCA. A.u. denotes arbitrary fluorescence units; bar graphs represent the mean. Error bars represent SD based on n=3 independent experiments.

(e). Viral tRNA operon-expressed tRNAs decode TCR codons as serine. The amino acid identity of the translated TCA codon within elastin₁₆ TCA-sfGFP-His6 was confirmed by tandem mass spectrometry from Syn61 3 cells containing the REP12 tRNA operon and its cognate promoter. The figure shows the amino acid sequence and MS/MS spectrum of the analyzed elastin₁₆ TCR peptide. MS/MS data was collected once.

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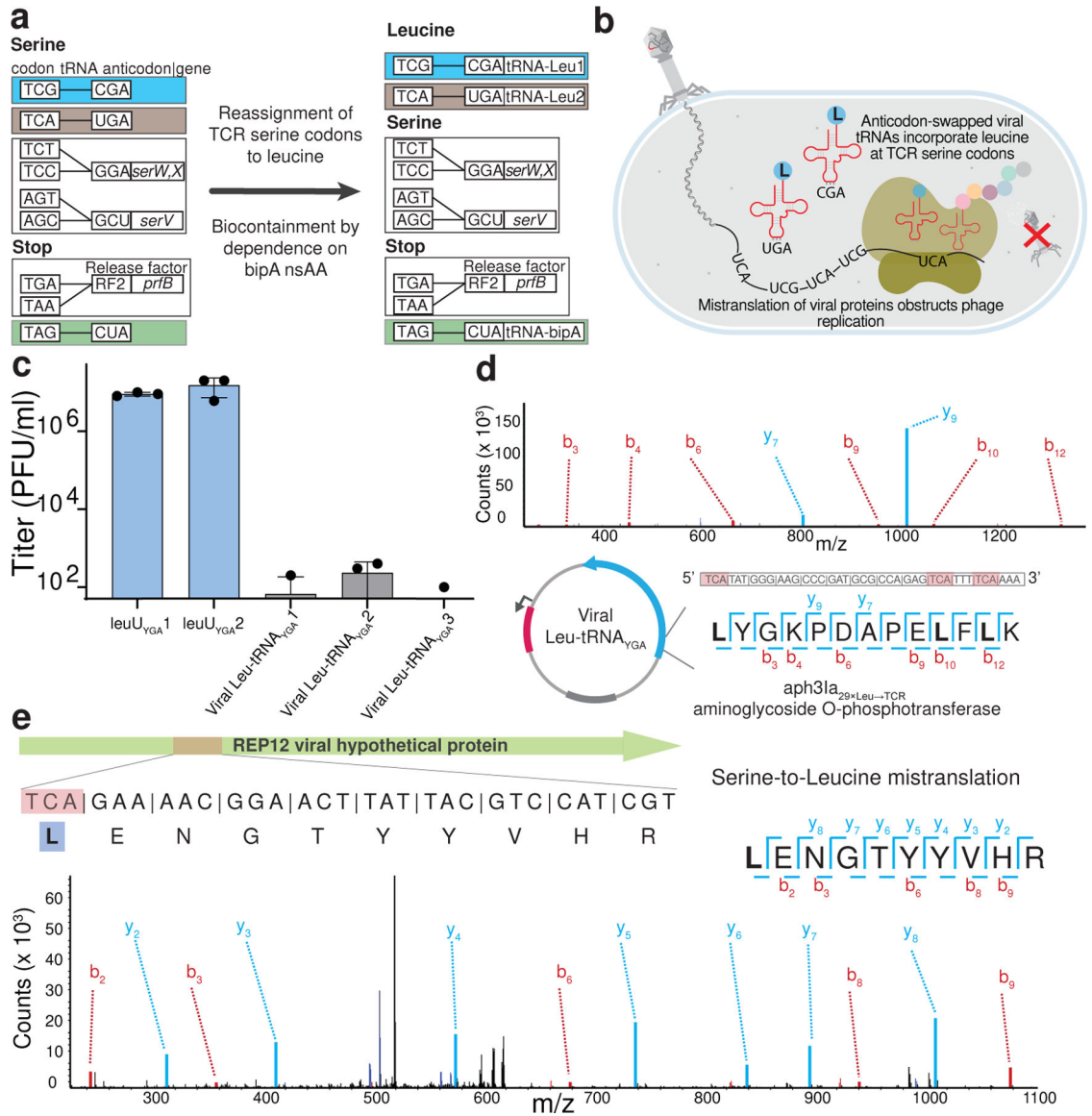


Figure 3. An amino-acid-swapped genetic code provides multi-virus resistance.

(a). The creation of an *E. coli* GRO, Ec_Syn61 3-SL, in which both TCA and TCG—naturally serine-meaning—codons are translated as leucine. The introduction of bacteriophage-derived Leu-tRNA_{UGA} and Leu-tRNA_{CGA} to Syn61 3 reassigns TCA and TCG codons to leucine, while the reassignment of the TAG stop codon to encode L-4,4'-biphenylalanine (bipA) in an essential gene of the host ensures the biocontainment of Ec_Syn61 3-SL.

(b). Schematic of viral infection in Ec_Syn61 3-SL. The reassignment of sense codons TCA and TCG to leucine in Ec_Syn61 3-SL provides multivirus resistance by mistranslating the viral proteome.

(c). Bacteriophage-derived Leu-tRNA_{UGA} and Leu-tRNA_{CGA} expression in Syn61 3 provides multivirus resistance. The figure shows the titer of lytic Syn61 3 phages following the infection of the corresponding Leu-tRNA_{YGA}-expressing Syn61 3 strain with a mixture

of twelve distinct REP Syn61-3 phages (Extended Data Table 1, Supplementary Data 3). All experiments were performed in three independent replicates; dots represent data from n=3 independent experiments; bar graphs represent the mean; the error bars represent SD.

(d). The reassignment of TCR codons to leucine within the coding sequence of *aph3Ia*_{29×Leu→TCR} in Syn61-3-LS was confirmed by tandem mass spectrometry. The figure shows the amino acid sequence and MS/MS spectrum of the detected *aph3Ia*_{29×Leu→TCR} peptide and its coding sequence. MS/MS data was collected once.

(e). Mistranslated viral protein synthesis in Ec_Syn61-3-SL. The figure shows the amino acid sequence and MS/MS spectrum of a bacteriophage-expressed protein, together with its viral genomic sequence, in which the naturally serine-coding TCA codon is mistranslated as leucine. The experiment was performed by infecting Ec_Syn61-3-SL cells, expressing Leu9-tRNA_{YGA} from *Escherichia* phage OSYSP, with the REP12 phage and the proteome of infected cells was analyzed by tandem mass spectrometry. MS/MS data was collected once.

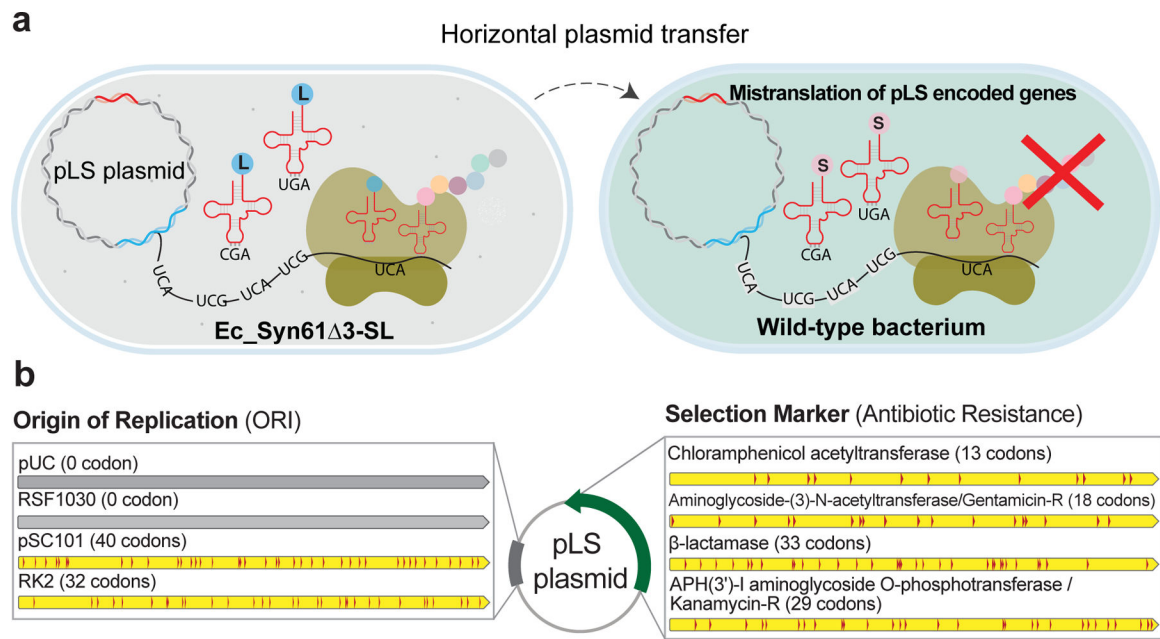


Figure 4. Addition of synthetic genetic information to a genetic code in which TCR codons encode leucine prevents horizontal gene transfer.

(a). We developed a set of plasmid vectors, termed the pLS plasmids, that rely on TCR codons to express leucine-containing proteins. pLS plasmids only function in Ec_Syn61 Δ 3-SL expressing bacteriophage-derived synthetic tRNA-Leu_{UGA} tRNAs, and the encoded proteins of pLS plasmids become mistranslated in cells bearing the canonical genetic code.

(b). The pLS plasmids offer multiple mutually orthogonal antibiotic resistance markers together with low to high copy-number origins-of-replication (ORI) that are addicted to an artificial genetic code in which leucine is encoded as TCR codons. Number in parenthesis marks the number of Leu_{TCR} codons in each gene. Detailed sequence information and the description of pLS plasmids are available in Supplementary Data 3.