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Genomic Recoding Broadly Obstructs the Propagation of Horizontally Transferred Genetic Elements

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SUMMARY

Horizontally transferred genetic elements such as viruses and conjugative plasmids move DNA between organisms, increasing genetic diversity but destabilizing engineered biological systems. Here we used a genomically recoded *Escherichia coli* strain lacking UAG stop codons and its recognition protein release factor 1 to study how an alternate genetic code influences horizontally transferred genetic element propagation. The alternate genetic code conferred resistance to multiple viruses (λ, M13, P1, MS2) at titers up to 10^{11} PFU/mL and impaired conjugative plasmids (F and RK2) up to 10^5 -fold. By recoding UAG codons to UAA in viruses and plasmids, we restored viral infectivity and conjugative function. Propagating viruses on a mixed community of cells with standard and alternate genetic codes reduced viral titer, and over time viruses adapted to the alternate genetic code. This work demonstrates that altering the genetic code broadly obstructs the propagation of horizontally transferred genetic elements and supports the use of genomic recoding as a strategy to stabilize engineered biological systems.

eTOC

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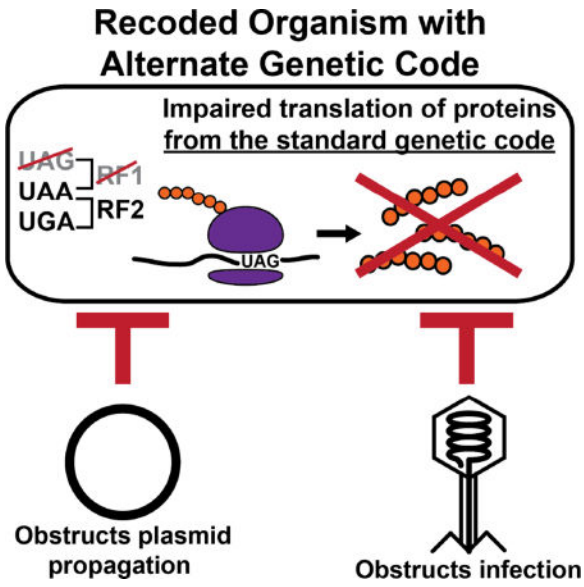
Author contributions

N.J.M. and F.J.I. conceived the study, designed experiments and wrote the paper. N.J.M. conducted experiments and F.J.I. supervised the study.



The conservation of the genetic code enables horizontal gene transfer, increasing genetic diversity but destabilizing engineered biological systems. Altering the genetic code obstructs the propagation of multiple viruses and conjugative plasmids, and motivates the use of genomic recoding as a strategy to genetically isolate and stabilize engineered organisms and microbial communities.

INTRODUCTION

Horizontal gene transfer (HGT), distinct from vertical transmission of genetic material from parents to offspring, is the intra-generational movement of genetic material between organisms and across species. It occurs through transformation, transduction, and conjugation, and often requires the presence of horizontally transferred genetic elements in the form of viruses and plasmids (Thomas and Nielsen, 2005). While HGT contributes to genetic diversity in nature (Ochman et al., 2000), it can introduce phenotypic changes that destabilize engineered biological systems through gene-gene interactions or global changes in cellular fitness (Baltrus, 2013), resulting in impaired growth or compromised functions. For example, biological-based production suffers losses from viral infection (Calendar, 2006), such as the 2009 infection of Genzyme's bioreactors that caused ~$300 million in losses of high-value therapeutics and alleged loss of human life (Bethencourt, 2009). Genes from horizontally transferred genetic elements can also compromise mechanisms designed to isolate and contain genetically modified organisms (GMOs) from natural species (Kong et al., 2008; Ronchel and Ramos, 2001). As biotechnology's societal and economic impact grows (Carlson, 2016), new approaches to minimize HGT and ensure the stability and safety of engineered biological systems are needed (Moe-Behrens et al., 2013; Schmidt and de Lorenzo, 2012).

Horizontally transferred genetic elements require expression of their genes to propagate, relying on the standard genetic code shared by most organisms to ensure accurate translation of their proteins. A genomically recoded *Escherichia coli* strain (C321.ΔA) containing an

alternate genetic code exhibited increased tolerance to infection with the bacteriophage T7 (Lajoie et al., 2013b), suggesting that an alternate genetic code could confer resistance to viral infection and more broadly, all types of horizontal gene transfer. While these preliminary results demonstrated a 25% attenuation of infection for phage T7, the earlier study did not examine broad resistance to viruses or explore other mechanisms of HGT such as conjugation. How the alternate genetic code could cause resistance and the role of recoded organisms in HGT resistance within microbial communities also remains unknown. The alternate genetic code of C321.ΔA was achieved by mutating all instances of the UAG stop codon to the synonymous UAA stop codon and deleting the gene encoding the release factor 1 (RF1) protein, which terminates translation at UAG codons (Isaacs et al., 2011; Lajoie et al., 2013b). We hypothesize that this alternate genetic code would obstruct HGT by compromising translation of UAG-ending genes in horizontally transferred genetic elements, impairing their propagation.

Here we assessed the ability of horizontally transferred genetic elements to propagate on the genomically recoded *E. coli* strain and find impaired infection by multiple viruses, plasmid conjugation, and plasmid propagation compared with control strains containing the standard genetic code. We then conducted rescue experiments with horizontally transferred genetic elements that had UAG codons mutated to UAA to determine which genes must be accurately translated to ensure propagation and establish a causal link between the alternate genetic code of C321.ΔA and resistance to HGT. To extend our investigations beyond the level of an individual host and to the level of microbial communities, we investigated viral fitness and capacity for adaptation on mixed populations of bacteria comprising alternate and standard genetic codes. Our findings demonstrate that an alternate genetic code confers broad resistance to horizontally transferred genetic elements and motivates the use of genomic recoding as a strategy to genetically isolate and stabilize engineered organisms and microbial communities for biological-based production and deployment of safe GMOs into open systems (*e.g.,* clinical medicine, environmental bioremediation).

RESULTS

Organisms with alternate genetic codes exhibit multi-viral resistance

We assayed a diverse panel of viruses that vary in proportion and type of UAG-ending genes (Table 1) for their ability to infect three strains of *E. coli* harboring standard or alternate genetic codes: (i) wild-type strain containing both UAG codons and RF1 (*+UAG+RF1*), (ii) strain lacking UAG codons but retaining RF1 (*ΔUAG+RF1*), and (iii) the C321.ΔA strain with an alternate genetic code lacking UAG codons and RF1 (*ΔUAGΔRF1*) (Figure 1A). Using serially-diluted phage to quantify plaque forming units per mL (PFU/mL), we found that viruses showed PFU/mL within 10-fold of each other on *+UAG+RF1* and *ΔUAG+RF1* hosts, but five (λ cI857, P1*vir*, P1 C1-100, M13, and MS2) of seven viruses produced no detectable plaques on *ΔUAGΔRF1* (Figure 1B, S1A, S1B, $p < 0.001$). The two phages able to infect *ΔUAGΔRF1* were Mu, which lacks UAG codons (Morgan et al., 2002), and T5, in which 12 of 13 UAG-ending genes are putative (Wang et al., 2005). Phage λ cI857, a temperature-sensitive variant of the lysogenic λ phage, produced clear plates on *ΔUAGΔRF1* at high titers (10^9 PFU/mL, n = 3) (Figure S1C), but no visible plaques at up to

10^7 PFU/mL (p = 1.9e-006). P1 *vir* and P1 c1-100, obligately lytic and temperature-inducible variants of the transducing phage P1 (Lobocka et al., 2004; Yarmolinsky, 1988), produced no plaques on *ΔUAGΔRF1* at any concentration, including undiluted phage titer at 10^9 PFU/mL (p = 2.5e-005 for P1 *vir*, p = 1.7e-006 for P1 c1-100). The *pF*-dependent phages MS2 and M13 (Loeb and Zinder, 1961; van Wezenbeek et al., 1980) also yielded no plaques on *ΔUAGΔRF1* at any concentration up to titers of 10^{11} (MS2, p = 3.8e-005) and 10^{12} (M13, p = 3e-4) PFU/mL, respectively.

While λ and P1 c1-100 were capable of adhering to *ΔUAGΔRF1*, they produced less than one progeny per cell infection (Figure S2), suggesting that the barrier to infection occurred after cell adhesion. To determine if restoring accurate translation of viral gene products can rescue infectivity, we used MAGE (Gallagher et al., 2014; Wang et al., 2009) to recode the four UAG-ending genes (*ea31, egrN, lgrQ, Rz*) in phage λ (Table S1, Figure S3A). This differs from gene deletion or mutagenesis of the coding region, since the coding region is unchanged and can produce the wild-type protein if placed in a host containing RF1. Phage λ variants propagated on *ΔUAGΔRF1* showed enrichment for UAG-to-UAA mutations, and 100% of phages propagated on *ΔUAGΔRF1* contained at least one UAG-to-UAA mutation in either *egrN* or *lgrQ* (Fig. 1C). To confirm the recovery of infection and determine which UAG-to-UAA reassignments were causative in restoring viral fitness, we infected all λ variants on *ΔUAG ΔRF1* and found that while recoding of the UAG codon terminating *egrN* to UAA partially restored viral titer (p = 1.2e-4), recoding of the UAG codon terminating *lgrQ* to UAA fully restored viral titer (p = 2.9e-6) on *ΔUAGΔRF1* to levels observed when infecting *+UAG+RF1* (Figure 1D, S3B, S3C). Both *egrN* and *lgrQ* encode proteins that promote host transcription of viral genes by preventing rho-dependent transcription termination (Grayhack et al., 1985; Schauer et al., 1987). These data suggest that recoding of the *lgrQ* stop codon from UAG-to-UAA could be necessary and sufficient to fully restore λ infection on *ΔUAGΔRF1.*

We next recoded M13 to create M13rec via PCR, mutating the UAG codon to UAA at the end of Gene IV, the sole UAG-ending gene that encodes the phage assembly protein (Table S1). We found that despite recoding, M13rec was unable to infect *ΔUAGΔRF1* (Figure 1E, S6, p = 3.0e-005). In viral adhesion and progeny per cell assays (Figure S3D, S3E), M13 bound all host strains similarly but showed one progeny per cell on *ΔUAGΔRF1* hosts, suggesting a failure to enter the cell. Additionally, phage MS2, which binds to the same surface receptor as M13, showed impaired binding on *ΔUAGΔRF1* hosts (p = 3.2e-4). This indicated a defect in the binding receptor for these phages, which is a conjugative pilus encoded by the plasmid *pF*, another horizontally transferred genetic element (Table 1) (Deng and Perham, 2002; Ou, 1973; Paranchych et al., 1971). Analysis of the *pF* sequence revealed ten UAG-ending genes (Table S1). Of these ten genes, one gene (*repE*) is involved in plasmid maintenance, two genes (*traY* and *traL*) are implicated in plasmid transfer, and one gene (*ybhA*) is a duplicate of an *E. coli* gene that forms a dimer, potentially producing dominant negative effects (Frost et al., 1994; Nelson et al., 1995; Watson et al., 1982). Using MAGE, we recoded the UAG codons terminating *repE, traY, traL*, and *ybhA* to UAA to produce a partially recoded *pF* (*pFpr*) and attempted infection with M13. We found that M13 was unable to infect *ΔUAGΔRF1* carrying *pFpr* (p = 0.0013), but when M13rec was infected

on *ΔUAGΔRF1* carrying *pFpr*, the phage recovered its ability to infect (Figure 1E, S3D, S3E, p = 1.8e-005).

The alternate genetic code obstructs the transfer and replication of conjugative plasmids

We next investigated the conjugation efficiency of *pF* in *ΔUAGΔRF1* to assess whether the alternate genetic code could obstruct forms of HGT beyond transduction. We quantified conjugative transfer efficiency of *pF* and *pFpr* from *+UAG+RF1, ΔUAG+RF1*, and *ΔUAGΔRF1* donors to *+UAG+RF1, ΔUAG+RF1*, and *ΔUAGΔRF1* recipients. Transfer of *pF* from *ΔUAGΔRF1* donors was below the 1% limit of detection for our assay (Figure 2A, p = 1.8e-6) and conjugation events were 100,000-fold less frequent than from *+UAG+RF1* cells (Figure S4A, p = 1.0e-4), while transfer of *pFpr* from the *ΔUAGΔRF1* donor exhibited a 2% transfer efficiency (Figure 2A, p = 3.7e-5). Recoding all 10 UAG codons to UAA (*pFrec*) resulted in a ~12-fold increase in transfer efficiency from *ΔUAGΔRF1* donors to 25% (Figure 2A, p = 4.8e-3), indicating UAG codons in *pF* impaired conjugative transfer. To more broadly investigate the effect of an alternate genetic code on conjugative plasmids, we also tested the ability of the broad host-range *pRK2* (Thomas and Smith, 1987) to transfer and replicate in *ΔUAGΔRF1* strains. We found that *ΔUAGΔRF1* experienced 25–50% reduction in conjugative transfer as a recipient and exhibited a 27% increase in doubling time (Figure 2B, S4B, Table S2, $p < 0.01$), which were recovered with UAG-to-UAA recoding in the plasmid, suggesting UAG codons in RK2 impaired both plasmid transfer and replication.

To determine if restoring accurate translation of conjugative plasmid gene products can rescue plasmid transfer and replication, we used MAGE to recode UAG-ending *pF* and *pRK2* genes to UAA and assayed propagation between and within cells by measuring conjugation rates and doubling times. After creating a library of *pF* variants with diverse UAG-to-UAA recoding in *+UAG+RF1* and *ΔUAGΔRF1* donors, we mated donors to a *+UAG+RF1* recipient to select for recovered conjugative function. All *pF* variants screened from *ΔUAGΔRF1* donors contained UAG-to-UAA mutations in *traY, traL*, or *repE*, of which 97.8% clones contained UAG-to-UAA mutations in *traY* or *traL* (Figure 2C) compared to only 55.3% of *pF* variants from *+UAG+RF1* donors. Assays of conjugative function confirmed that UAG-to-UAA mutations in *traY* and *traL* recover conjugative efficiency 10,000-fold (Figure S4A, p = 2.0e-4). In contrast, the MAGE-derived *pRK2* library conjugated into both *+UAG+RF1* and *ΔUAGΔRF1* recipients identified a single gene, *trfA*, which restored both conjugative function and host fitness. Gene *trfA* encodes proteins that initiate vegetative replication in *pRK2* (Pansegrau et al., 1994). In *ΔUAGΔRF1* recipients, 100% of *pRK2* variants contained a UAG-to-UAA mutation in *trfA*, compared to 23.4% from *+UAG+RF1* recipients (Figure 2D). Recoding *trfA* restored both conjugative efficiency and propagation of *pRK2* in *ΔUAGΔRF1* to levels seen in *+UAG+RF1* (Figure 2B, S4B, Table S2, p = < 1.0e-4). These results demonstrate that an alternate genetic code can impair replication and conjugation of plasmid-based horizontally transferred genetic elements, impeding their propagation within and between hosts.

Recoded organisms reduce viral titer in mixed microbial communities and prompt adaptation to the alternate genetic code

We examined how the presence of recoded organisms in a microbial community affects viral fitness by propagating phage λ on communities comprising varying ratios of two types of cells: permissive hosts with standard genetic codes and non-permissive hosts with alternate genetic codes (Figure 3A). As we increased the proportion of non-permissive hosts to permissive hosts, we expected viral titer to decrease as the virus is increasingly likely to bind and infect non-permissive hosts. We found that in the presence of 90 to 100% *ΔUAGΔRF1*, viral populations that start at 10^9 PFU/mL decrease continuously and are extinct by days five and three, respectively (Figure 3B, $p < 0.001$). In contrast, viral populations propagated on communities comprising 0%, 10%, or 50% *ΔUAGΔRF1* all showed increases in viral titer to 10^{10} on day one and then diverged. The populations of viruses propagated on 50% *ΔUAGΔRF1* declined on days two and three, stabilizing at a PFU/mL of 5- to 10-fold less than viral populations propagated on 10% or no *ΔUAGΔRF1* ($p < 0.05$).

To determine whether horizontally transferred genetic elements could adapt to the alternate genetic code, we subjected the highly mutagenic ssRNA phage MS2 (1.5×10^{-3} substitutions per bp per replication) (Drake, 1993) to selective pressure by propagating it on a microbial community containing changing ratios of strains *ΔUAG+RF1* and *ΔUAGΔRF1* carrying *pFpr* (Figure 3A). After five days of propagation in soft agar, the MS2 population produced opaque plaques when infected on only *ΔUAGΔRF1* carrying *pFpr*. Sequencing of eight plaques revealed that phages contained the following two mutations (designated as MS2rec): (1) a UAG-to-UAA mutation in the last codon of the *rep* gene, and (2) a nonsense AGA-to-UGA mutation in the penultimate codon of the *mat* gene to create a premature stop codon (Figure 3C) (Fiers et al., 1976). These mutations eliminate UAG codon use and provide direct experimental evidence that viruses adapt their genetic code to achieve compatibility with the alternate genetic code of the *ΔUAGΔRF1* host. To improve plaque clarity, we further evolved MS2rec on decreasing ratios of *ΔUAG+RF1* to *ΔUAGΔRF1* until phage produced clearer plaques when infected on *ΔUAGΔRF1*. We sequenced three clones from this population and found two clones contained an A->C transversion in the overlapping lysis and coat protein genes and designated this phage MS2rec2. MS2rec2 demonstrated a 10^9 increase over MS2 in ability to infect hosts with an alternate genetic code in the presence of *pFpr* (Figure 3D, $p = 0.026$), recovering infection to within 10-fold of MS2 on hosts with standard genetic codes and demonstrating viral adaptation to the alternate genetic code.

DISCUSSION

Horizontally transferred genetic elements have driven both the evolution of natural mechanisms to restrict HGT such as restriction enzymes and CRISPR-Cas (Thomas and Nielsen, 2005), but these mechanisms are often sequence-specific. Additionally, anthropogenic strategies to reduce HGT exist (Getino et al., 2015), but these require continuous input of a small molecule to prevent HGT from occurring. In this study, we demonstrate that altering the genetic code of a cell can reduce HGT by broadly obstructing propagation of horizontally transferred genetic elements in a host and within microbial

communities. Despite the rarity of UAG codons (Table 1), an alternate genetic code conferred resistance to various viruses with different life cycles (lytic, lysogenic, and non-lytic) and conjugative plasmids containing selectively advantageous genes (*e.g.,* antibiotic resistance). Additionally, in the F-dependent phages M13 and MS2, restoring viral infection required recoding UAG codons in both the viral genome and *pF*, suggesting a two-layer model of immunity against F-dependent viruses that may extend to other viruses dependent on conjugative plasmids for infection.

Recoding UAG codons to UAA in phages and conjugative plasmids identified key causative genes and established that UAG codons caused impaired propagation. We found that a single UAG codon could serve as a barrier to propagation of both viruses (λ, M13) and conjugative plasmids (RK2). Given that 93% of all Enterobacteria and *Escherichia* phages on NCBI's Viral Genomes Resource have at least one UAG-ending gene (Table S3) (Brister et al., 2015), our data suggest that the alternate genetic code lacking UAG assignment may confer broad multiviral resistance. Though the molecular mechanism of this impairment has not been experimentally investigated in this study, we hypothesize that the lack of RF1 causes ribosomal stalling at UAG and results in two possible outcomes that would cause impaired propagation: (1) tmRNA-mediated rescue that results in degradation of the translated peptide (Keiler, 2015), or (2) near-cognate or amber suppression (Eggertsson and Soll, 1988), creating a C-terminal tail that disrupts protein function.

Our study also demonstrates that alternate genetic codes impair viral population fitness in microbial communities, reducing viral titer and prompting emergence of viral variants that have adapted to the alternate genetic code. In microbial communities, the presence of organisms with alternate genetic codes reduced λ viral titer when they comprised at least half of the host population, and we interpret this as a reduction in viral population fitness. Previous work demonstrated that non-permissive host cells overexpressing viral surface receptors could reduce viral titer when mixed with permissive host cells (Dennehy et al., 2007), as viral binding kinetics favor attachment and attempted infection of non-permissive hosts. However, we did not modify surface receptor expression in our ΔUAGΔRF1, suggesting that alternate genetic codes can reduce viral titer and viral population fitness in a microbial community more broadly than surface receptor-based strategies. Furthermore, over time the ssRNA phage MS2 mutated away from UAG codon use when propagated in the presence of the alternate genetic code, indicating that viruses experience a selective pressure to reduce the presence of UAG codons in their genomes.

From an applied perspective, the ability to impair propagation of horizontally transferred genetic elements provides a useful strategy for genetic isolation in diverse biomanufacturing and biocontainment applications. Utilizing organisms with alternate genetic codes in industrial settings could increase stability and reduce the risk and cost of biological production (Calendar, 2006). In GMOs that function as engineered probiotics (Steidler, 2003) or in bioremediation applications (Pieper and Reineke, 2000), alternate genetic codes can act as barriers to gene transfers that could compromise biocontainment mechanisms such as natural or synthetic auxotrophies (Mandell et al., 2015; Rovner et al., 2015; Steidler et al., 2003) and toxin kill switches (Cai et al., 2015; Chan et al., 2016; Gallagher et al., 2015). While a virus adapted to the alternate genetic code with few mutations (Figure 3C),

we anticipate that greater barriers achieved by further recoding within *E. coli* and additional organisms could prove insurmountable to horizontally transferred genetic elements. Research into sense codon reassignment is already underway in *E. coli* and other bacterial species (Krishnakumar et al., 2013; Lajoie et al., 2013a), paving the way for alternate genetic codes with multiple codon reassignments. By expanding recoding efforts to multiple species, we envision the development of synthetic microbial communities with alternate genetic codes that are genetically isolated and robust to perturbation by HGT. In assaying the ability of horizontally transferred genetic elements to utilize a host with an alternate genetic code, we step into an ancient evolutionary arms race between selfish genetic elements and the hosts they exploit. This interplay has generated a panoply of adaptations in the history of life (Syvanen, 2012) and driven the evolution of microbial defense systems against horizontally transferred genetic elements (Bickle and Kruger, 1993; Makarova et al., 2011). Since metagenomic studies have revealed that alternate genetic codes are more prevalent than previously thought (Ivanova et al., 2014) and reassignments of the same codons have occurred in multiple lineages (Knight et al., 2001), some have suggested that alternate genetic codes could serve as defense systems against horizontally transferred genetic elements (Shackelton and Holmes, 2008). While previous research into codon usage showed that synthesis of viral genes utilizing underrepresented codon pairs reduced the virulence of both poliovirus and influenza virus (Coleman et al., 2008; Nogales et al., 2014), our work demonstrates that an alternate genetic code can impede horizontally transferred genetic element propagation and create barriers to HGT. These results support the hypothesis that alternate genetic codes act as defense against exploitation by horizontally transferred genetic elements, suggesting an evolutionary interplay between the genetic code and horizontal gene transfer that may have driven the evolution of alternate genetic codes.

Experimental Procedures

Viral Relative Titers

To quantify relative titers, we mixed 100-fold dilutions of phage with 300 μL of mid-log (OD_{600}=0.5) cells in 3 mL of TK soft agar and poured onto TK solid agar plates. We found that MS2 produced clearer plaques if propagated on late log (OD_{600} = 1.0) cells and used this OD instead. We plated each dilution in triplicate, incubated plates overnight, and counted plaques the next day.

Recoding of Viral Genomes

To recode lambda phage UAG codons to UAA, we lysogenized λ cI857 in an MG1655 strain with a *mutS* deletion and a constitutively expressed lambda beta protein on a plasmid. At mid-log, we rinsed cells, re-suspended them in oligo, and electroporated using the methods described previously (Gallagher et al., 2014). We then allowed cells to return to mid-log density and repeated for a total of 5 MAGE cycles. The oligonucleotide sequences we used to convert UAG codons to UAA are available in the **Oligonucleotides** Excel spreadsheet. After MAGE, we induced lytic cycle by shifting an aliquot of cells to 42 °C for 20 minutes, and then plated 100-fold serial dilutions of this in the same manner as viral relative titers on *+UAG+RF1* or *ΔUAGΔRF1*. We then assayed 47 plaques from each host for UAG-to-UAA mutations via sequencing.

We utilized PCR with a UAG-to-UAA mutation in primer overhangs to recode the M13 genome. To minimize error in replication, we used High-fidelity PCR Mix from Kapa Biosystems (KK2602). After PCR, we circularized linear amplified phage genome by mixing 100–300 ng of DNA in Gibson Assembly Master Mix from NEB and incubating for 1 hour at 50°C. We then drop-dialyzed the assembly for 1 hour to remove salts and transformed phage genome into *+UAG+RF1* with *pF* to extract virus. We sequenced isolated virus to verify the UAG-to-UAA mutation and to detect any accessory mutations.

Microbial community assays

Microbial community assays were performed using three phage populations as biological replicates propagated on mixed populations of *ΔUAG+RF1* (viable host) and *ΔUAGΔRF1* (nonviable host) cells at varying ratios. *ΔUAG+RF1* was chosen instead of *+UAG+RF1* because its doubling time is similar to *ΔUAGΔRF1*, simplifying cell ratio calculations. Each day, we extracted phage as described above, diluted viral population 10^4, and re-infected on fresh mixed populations of cells with the same ratio. We titered phage using methods described above.

We evolved MS2 via serial propagation on mixed populations of *ΔUAG+RF1* (viable host) and *ΔUAGΔRF1* (nonviable host) cells. *ΔUAG+RF1* was chosen instead of *+UAG+RF1* because its doubling time is similar to *ΔUAG ΔRF1*, simplifying cell ratio calculations. Each day, we extracted phage as described above, diluted viral population to get 10^4 to 10^5 plaques, and re-infected on mixture of strains. We propagated MS2 on agar overlays with 1:1 ratio of *ΔUAG+RF1* to *ΔUAGΔRF1* cells for 5 days, when we plated viral populations on only *ΔUAGΔRF1* cells and sequenced 8 resulting plaques. We further evolved MS2rec using the following propagation: 2 days 1:1 ratio *ΔUAG+RF1* to *ΔUAGΔRF1*, 4 days 1:3 ratio *ΔUAG+RF1* to *ΔUAGΔRF1*, 2 days 1:9 ratio *ΔUAG+RF1* to *ΔUAGΔRF1*, 1 day on *ΔUAGΔRF1* only. We picked 3 plaques from this population for sequencing.

Recoding of conjugative plasmids and quantifying selective pressure on UAG->UAA mutations

To recode conjugative plasmids, we mated plasmids into cells carrying the lambda red cassette and performed MAGE as described previously (Gallagher et al., 2014) with UAG-to-UAA mutation oligonucleotides whose sequences are available in the **Oligonucleotides** Excel spreadsheet. For *pF*, we performed 8 cycles of MAGE in *ΔUAGΔRF1* and *+UAG +RF1* backgrounds and then conjugated to *+UAG+RF1* strains to identify mutations that recover conjugative ability. For *pRK2*, we performed 10 cycles of MAGE in a *+UAG+RF1* background and then conjugated to *+UAG+RF1* or *ΔUAGΔRF1* strains to identify mutations that recover plasmid maintenance and conjugative efficiency. In both cases, the clone with the most UAG-to-UAA mutations was then chosen for subsequent cycles of MAGE until all UAG codons were converted to UAA.

Quantifying Conjugation Efficiency

We used conjugation conditions described previously (Ma et al., 2014). Briefly, we grew cultures of donor and recipient cells to late log in antibiotics selecting for plasmid or recipient and then rinsed and re-suspended in media to remove antibiotics. We then

concentrated cells and normalized to OD_{600}=20 by doing 100-fold dilution and normalizing to OD_{600}=0.2, then mixed donors and recipients in 1:1 ratio and spotted onto pre-warmed LB Lennox agar plates in 2× 20uL and 6× 10uL pattern. We incubated plates at 37°C for 1 hour (*pRK2*) or 2 hours (*pF*), then rinsed cells from the plate, diluted serially 10-fold, and plated on plates containing antibiotic selecting for recipient strain and incubated overnight at 37°C. We then picked 86 colonies from plates selecting for the recipient strain and patched them onto plates selecting for recipient strain with the conjugative plasmid, incubated plates overnight at 37°C, and counted the number of patched colonies that grew.

Statistical Analysis

P values were calculated using unpaired T-tests in GraphPad Prism 6 with no assumption of consistent standard deviation and a false discovery rate threshold of 0.01.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- An alternate genetic code obstructs the propagation of viruses and conjugative plasmids
- Recoding viruses and plasmids to match the altered genetic code restores propagation
- Recoded organisms reduce viral population fitness within microbial communities
- Viruses adapt to match the alternate genetic code and infect recoded organisms

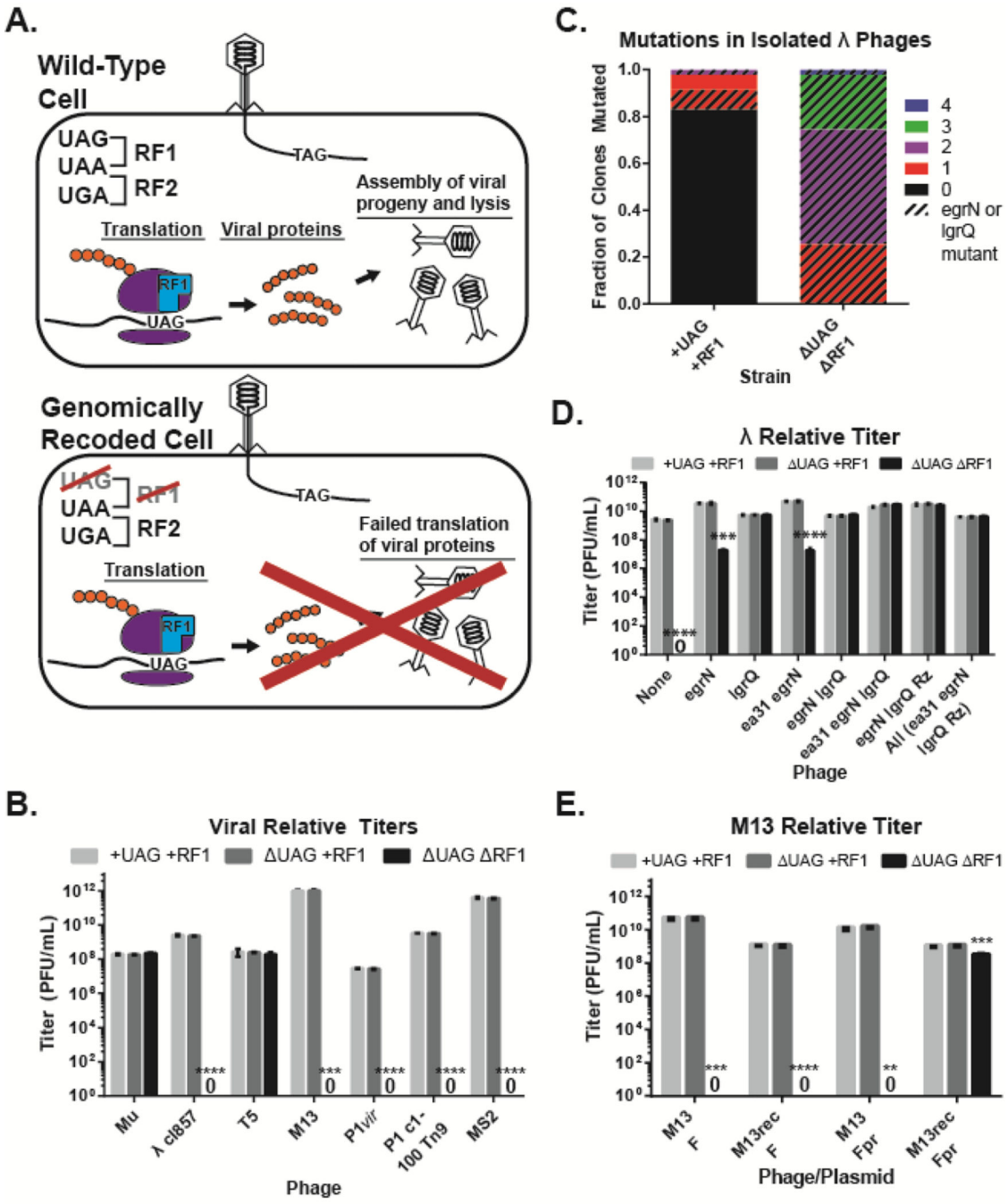

Figure 1. The alternate genetic code obstructs viral infection

(A) Schematic depicting viral infection of cells with standard genetic codes or alternate genetic codes with no assigned meaning for the UAG codon. (B) Relative titers of viruses on strains +*UAG+RF1, ΔUAG+RF1*, and *ΔUAGΔRF1*. (C) Mutation analysis of 94 λ plaques isolated after recoding using MAGE. Colors represent number of mutations and the bar pattern represents proportion of mutants with UAG-to-UAA recoding in *egrN* or *lgrQ*. (D) Relative titers of λ phages with varying recoded loci (x-axis). (E) Relative titers of wild-type and recoded M13 phages infected on hosts with wild-type or partially recoded (Fpr) *pF*. For

all relative titers, data are mean with standard deviation, n=3. “0” indicates zero plaque forming units (PFU)/mL. P-values are as follows: * is $P \leq 0.05$, ** is $P \leq 0.01$, *** is $P \leq 0.001$, and **** is $P \leq 0.0001$.

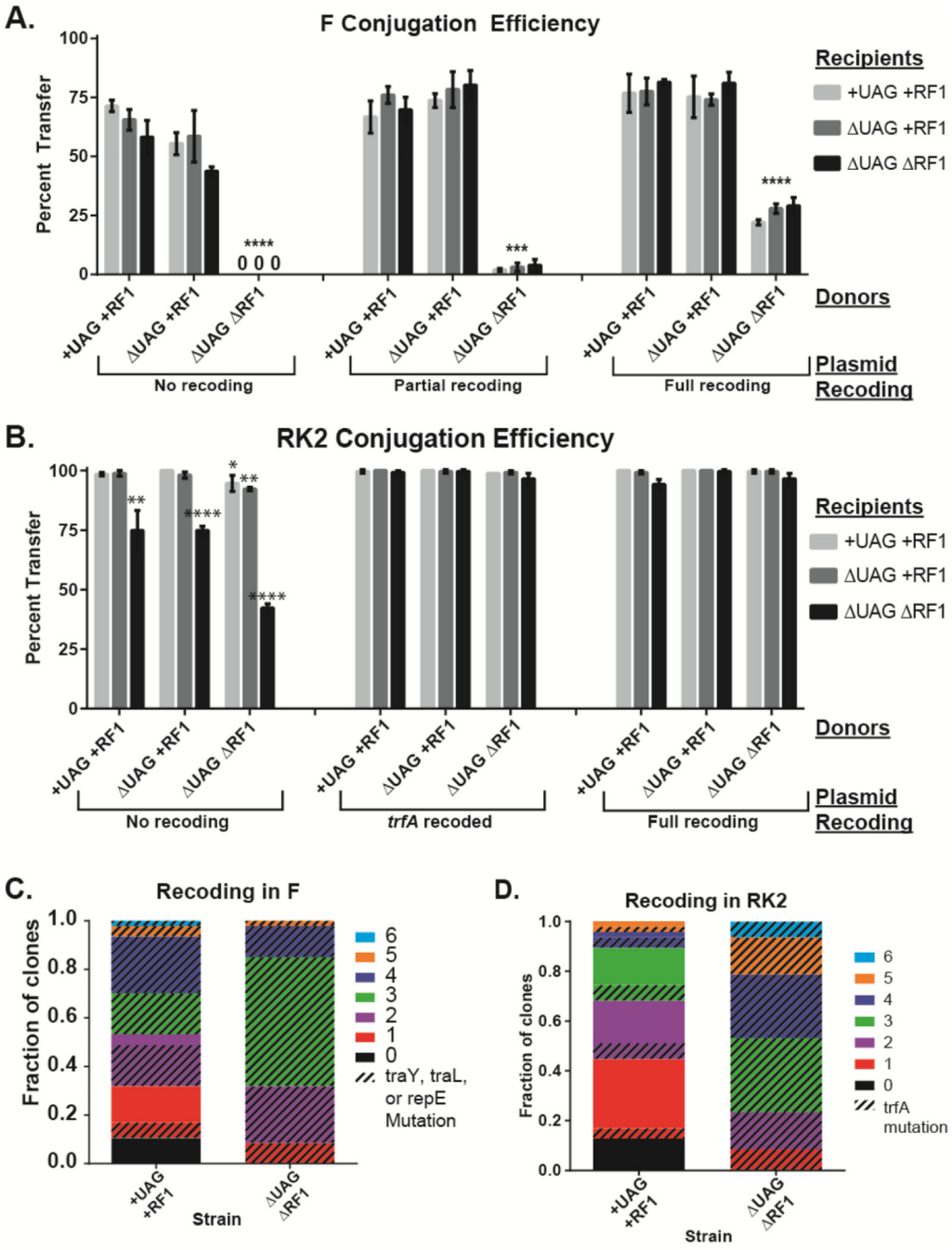

Figure 2. The alternate genetic code obstructs conjugation

Conjugation efficiency from donors with standard and alternate genetic codes (x-axis) to recipients with standard and alternate genetic codes (bars) for wild-type and recoded (A) *pF* and (B) *pRK2* conjugative plasmids. "0" indicates transfer efficiency was below limit of detection of 1%. Data are mean with standard deviation, n=3. P-values are as follows: * is P ≤ 0.05, ** is P ≤ 0.01, *** is P ≤ 0.001, and **** is P ≤ 0.0001. (C) Mutation analysis of 96 *pF* variants isolated after recoding using MAGE and conjugation from *+UAG+RF1* and *ΔUAGΔRF1*. (D) Mutation analysis of 96 *pRK2* variants isolated after recoding using

MAGE and conjugation to *+UAG+RF1* or *ΔUAGΔRF1*. For mutation analysis, colors represent number of mutations and pattern represents mutants with UAG-to-UAA recoding in indicated genes.

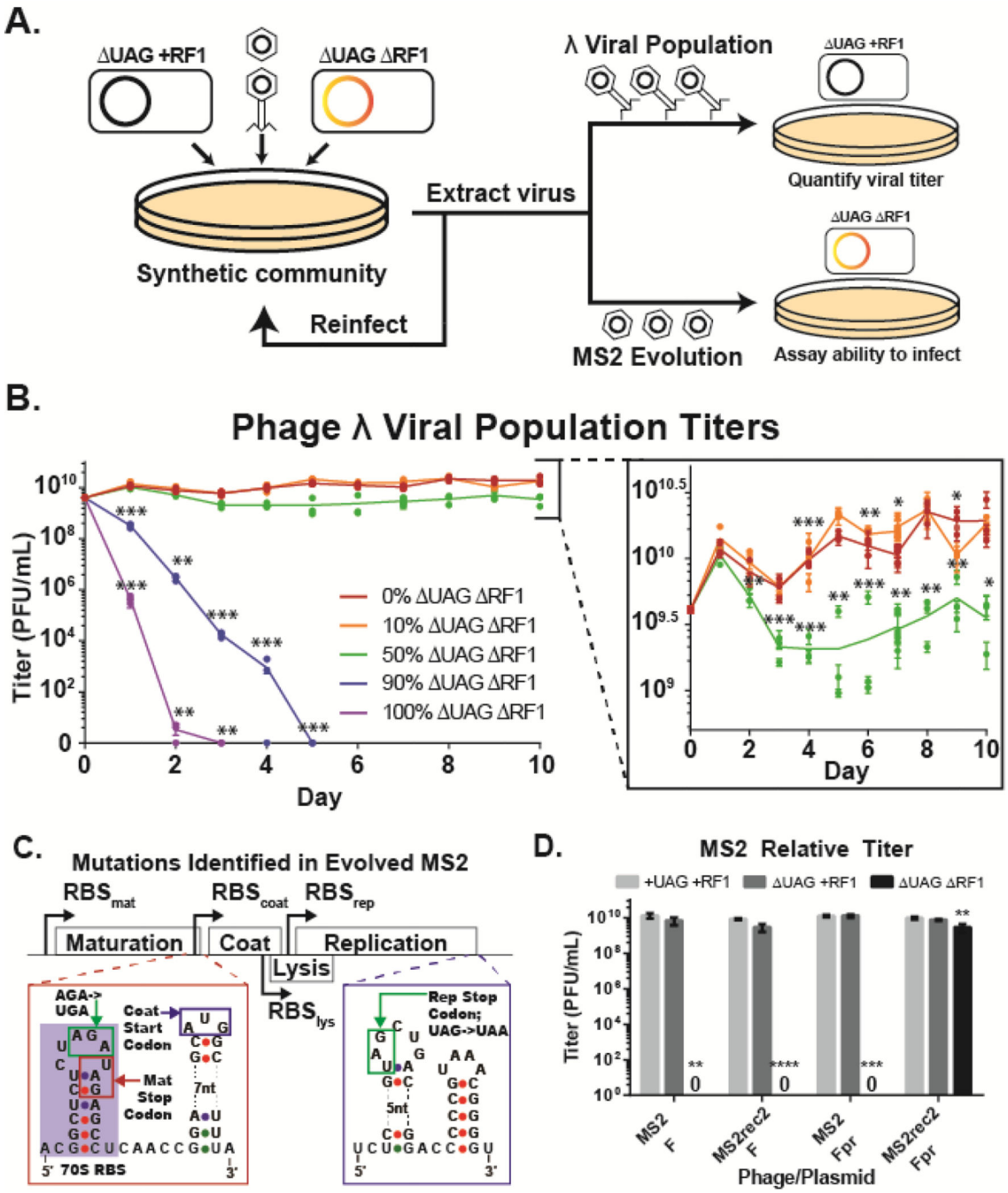

Figure 3. Recoded organisms reduce viral population fitness in microbial communities and select for viral mutations that eliminate UAG codon use

(A) Schematic of microbial community assays. Phages are infected on a co-culture containing varying ratios of *ΔUAG+RF1* and *ΔUAGΔRF1*, extracted the next day, and propagated on a co-culture with the same cell ratio. Viral populations of λ were quantified by infection on *ΔUAG+RF1,* and ability of phage MS2 to infect *ΔUAGΔRF1* was assayed by plating on *ΔUAGΔRF1* containing *pFpr*. (B) Titers of phage λ viral populations propagated on microbial communities containing cells with standard and alternate genetic codes. Lines are mean of 3 biological replicates for each population. (C) Location of mutations

eliminating UAG codon usage in the MS2 genome (Calendar, 2006; Fiers et al., 1976). (D) Relative titers of wild-type and recoded MS2 (MS2rec2) phages infected on *ΔUAGΔRF1* with *pF* or *pFpr*, which is required for phage infection. Data are mean with standard deviation, n=3. P-values are as follows: * is $P \leq 0.05$, ** is $P \leq 0.01$, *** is $P \leq 0.001$, and **** is $P \leq 0.0001$.

Table 1

Horizontally transferred genetic elements used in this study

Name	Type	Genome Type	Size (kb)	# UAG-ending/Total genes	Adhesion Site
Mu	Virus	dsDNA	36.7	0/55	Lipopolysaacharide component
λ cI857	Virus	dsDNA	48.5	4/92	Maltoporin (lamB)
T5	Virus	dsDNA	121.8	13/195	Ferrichrome receptor (fhuA)
M13	Virus	ssDNA	6.4	1/10	*pF* conjugative pilus
P1*vir*	Virus	dsDNA	94.0	12/116	Lipopolysaacharide component
P1 c1-100	Virus	dsDNA	94.5	13/117	Lipopolysaacharide component
MS2	Virus	ssRNA	3.6	2/4	*pF* conjugative pilus
pF	Conjugative Plasmid	dsDNA	100.3	10/106	N/A
pRK2	Conjugative Plasmid	dsDNA	60.1	9/74	N/A