Bacteriophages use an expanded genetic code on evolutionary paths to higher fitness

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Bioengineering advances have made it possible to fundamentally alter the genetic codes of organisms. However, the evolutionary consequences of expanding an organism’s genetic code with a noncanonical amino acid are poorly understood. Here we show that bacteriophages evolved on a host that incorporates 3-iodotyrosine at the amber stop codon acquire neutral and beneficial mutations to this new amino acid in their proteins, demonstrating that an expanded genetic code increases evolvability.

The evolution of life on Earth has been dictated by the underlying constancy of a nearly universal genetic code with twenty amino acids. However, examples of natural genetic codes that have been functionally expanded with a twenty-first amino acid and the multitude of known post-translational protein modifications suggest that, though aspects of the genetic code have been optimized by evolution, it does not provide the necessary chemical diversity to best perform all functions of potential benefit to organisms. Technologies exist to augment genetic codes with a noncanonical amino acid (ncAA) by introducing an orthogonal aminoacyl-tRNA synthetase (aaRS) and a cognate tRNA recognizing the amber stop codons. We hypothesized that organisms given the ability to encode a twenty-first amino acid would evolve to use this new chemical building block on mutational pathways to higher fitness.

Several challenges can arise when attempting to evolve an organism with a newly expanded genetic code, and the directed evolution of even single proteins with ncAAs has been limited to date. The organism of interest must first survive the globally disruptive change in how its genomic information is decoded into its protein. Next, the reassigned codon must be translated with sufficient efficiency and fidelity for substitutions of the new codon to be beneficial, which is less likely if translating this codon sometimes results in truncated proteins or ambiguous amino acid incorporation. Evolution may also need to proceed for many generations to observe ncAA substitutions because only a small fraction of mutations will result in changes to the reassigned codon. Finally, one must circumvent rejection of the reengineered genetic code in cases where mutations that lead to the ncAA no longer being incorporated must circumvent rejection of the reengineered genetic code in cases where readthrough of a reassigned stop codon is deleterious, indicating that some phages in these populations had evolved amber codons in important or essential genes, these mutations are expected to result in addiction, such that an organism requires an alternative genetic code for viability. To test for this level of dependence, we titered the evolved T7 populations on three E. coli hosts, which either terminate translation (BL21(DE3)) or incorporate 3-iodotyrosine (RF0 IodoY) or tyrosine (RF0 Tyr) at the amber codon (Fig. 1b). All of the populations produced statistically indistinguishable numbers of phage plaques on RF0 IodoY and RF0 Tyr cells, but one WT and three Δ2 populations produced significantly (P ≤ 0.01) fewer plaques on the BL21(DE3) host (Fig. 1c), indicating that some phages in these populations had evolved amber codons inside key genes.

To determine exactly what genetic changes had taken place, we sequenced DNA samples isolated from four evolved T7 populations. Despite an approximately tenfold difference in mutation rates, the base substitution spectra in Δ2 and WT populations were similar (Supplementary Fig. 4). However, the overall character of genome evolution shifted from directional selection in the wild-type populations (dN/dS = 1.58) to purifying selection in the hypermutator populations (dN/dS = 0.57). Deletions overlapping gene 0.7 that are known to be beneficial were present, in aggregate, in ~100% of every sequenced population (Supplementary Fig. 5 and Supplementary Table 1), and there was a deletion overlapping two hypothetical genes in one population (Supplementary Fig. 6).

Among the point mutations present in these populations (Fig. 1d), we observed two substitutions of amber codons that reached high frequencies: one in T7 RNA polymerase (RNAP) and the other in the T7 type II holin. Patching random phage isolates from the
respective populations confirmed that each new amber codon was associated with a defect in forming plaques on the BL21(DE3) normal genetic code E. coli host. As anticipated, we also observed compensation for readthrough of phage proteins ending in the amber codon. Notably, mutations appeared to restore T7 exonuclease (gene 6) termination in close to 100% of all four sequenced populations, respectively.

![Image]

**Figure 1 | Genome evolution of a bacterial virus with a newly expanded genetic code.** (a) In the evolution experiment, six populations each of WT and Δ2 bacteriophage T7 were passaged on a release factor 1 knockout (RF0) E. coli host strain carrying an orthogonal tRNA and aAR5 system that recodes the amber stop codon to the non-natural amino acid 3-iodotyrosine. (b) E. coli host strains used to test whether evolved phages required amber suppression by 3-iodotyrosine or tyrosine to replicate. (c) Relative titer (number of plaques) formed by each evolved bacteriophage population on the host strains in b. Values are the average of three technical replicates with error bars that represent 95% confidence limits. P values for reduced phage number on the alternative hosts compared to RF0 IodoY are for Student’s t-tests assuming equal variance and using the Bonferroni correction for multiple testing. (d) Locations of point mutations with frequencies ≥5% observed in metagenomic DNA sequencing data for the four bacteriophage populations boxed in c. Red open reading frames encode T7 proteins with known functions. Mutations of interest in regions highlighted in yellow are discussed in the text and following figures. There were roughly 50 and 400 mutations present with frequencies ≥1% in the evolved WT and Δ2 T7 populations, respectively.

observations indicate that type II holin activity was under strong directional selection during the evolution experiment. The amber mutation in the T7 type II holin occurs with the highest frequency (53%) of any coding base substitution in this population and with the highest frequency of any gene 17.5 mutation in any sequenced phage population. This result is even more notable given that it is in a wild-type rather than a hypermutator background. As expected for a highly beneficial mutation, phages with the amber lyse RF0 IodoY cells more completely at earlier times than phages from the same population without it, and this difference is not found on the RF0 Tyr host, where tyrosine incorporation at the amber codon restores the normal type II holin sequence (Supplementary Fig. 9).

To directly test whether the 3-iodotyrosine substitution in the type II holin protein was beneficial to phage fitness, we isolated spontaneous rescue mutants of a phage with this amber that could plaque on normal genetic code cells. Only tryptophan and tyrosine mutants were obtained, suggesting that position 39 of the holin is constrained to aromatic amino acids (Fig. 2b). The evolved isolate coding 3-iodotyrosine was more fit than the rescue mutants with tyrosine or tryptophan at this position when they were competed with the amber lyse RF0 IodoY cells more completely at earlier times than phages from the same population without it, and this difference is not found on the RF0 Tyr host, where tyrosine incorporation at the amber codon restores the normal type II holin sequence (Supplementary Fig. 9).

In contrast, several lines of evidence indicate that the Tyr39-to-amber substitution in the T7 type II holin (gene 17.5) is beneficial to phage fitness (Fig. 2a). Holins regulate lysis timing by forming pores in the bacterial membrane through which lysozyme degrades the cell wall23, and optimizing lysis time is critical to maximizing phage progeny2,23. We found a higher density of point mutations with frequencies ≥1% within gene 17.5 than in the genome at large (binomial test; P = 1.5 × 10^{-4}). Furthermore, all 22 of these base substitutions in the type II holin were nonsynonymous (Supplementary Table 2), and nearly 100% of phages in each WT and >50% in each Δ2 population have a mutation somewhere in gene 17.5. Together these
Brief communication

E. coli optimality of the natural genetic code and to discover unexpected
makes it possible to experimentally address questions about the
180
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the ancestral amino acid and a chemically similar amino acid tolerated at
indicating that 3-iodotyrosine confers a fitness benefit compared to both
that the amber codon increases in frequency relative to the alternative,
(TGG) at that position. In each competition, lysates of the two phage
competed against rescue mutants with a tyrosine (T ac) or tryptophan
with the amber codon (TaG) at position 39 of the holin protein was
observed. The numbers of rescue mutants found with each codon
eight possible neighboring codons accessible by single-base mutations
with the standard genetic code and isolating fast-growing plaques. of the
this amber mutation were obtained by plating on
suggest that this mutation increases phage fitness on the rF0 IodoY
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(Signatures of molecular evolution described in the text strongly
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E. coli host. (b) Rescue mutants of an evolved WT L6 phage isolate with
this amber mutation were obtained by plating on E. coli BL21(DE3) hosts
with the standard genetic code and isolating fast-growing plaques. Of the
the eight possible neighboring codons accessible by single-base mutations
that code for amino acids, only tyrosine and tryptophan mutants were
observed. The numbers of rescue mutants found with each codon
are indicated at the end of the arrows. (c) The evolved phage isolate
with the amber codon (TAG) at position 39 of the holin protein was
competed against rescue mutants with a tyrosine (TAC) or tryptophan
(TGG) at that position. In each competition, lysates of the two phage
preconditioned separately were mixed and then passaged on RFO IodoY
cells for three transfers. Sanger sequencing traces at this position show
that the amber codon increases in frequency relative to the alternative,
indicating that 3-iodotyrosine confers a fitness benefit compared to both
the ancestral amino acid and a chemically similar amino acid tolerated at
this position.

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Methods

Methods and any associated references are available in the online
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Figure 2 | Beneficial amber mutation in the T7 holin II protein.
(a) The frequency of an amber mutation resulting in a substitution from
tyrosine to 3-iodotyrosine in the T7 type II holin (gene 17.5) can explain
the reduced titer of population WT L6 on the standard genetic code host
(Fig. 1c). Signatures of molecular evolution described in the text strongly
suggest that this mutation increases phage fitness on the RFO IodoY
E. coli host. (b) Rescue mutants of an evolved WT L6 phage isolate with
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this position.

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Author contributions

M.J.H., J.W.E., J.E.B. and A.D.E. conceived the study. M.J.H. performed evolution
experiments. J.E.B. and M.J.H. analyzed sequencing data. M.J.H. and J.W.E. created
RFO Tyr and characterized phage lysis times. D.R.B. and E.M.M. performed proteomics
data. J.E.B. performed statistical analyses. J.E.B., M.J.H., J.W.E. and D.R.B. created
figures and wrote the manuscript. All of the authors designed experiments and
edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available in the online version of the paper. Reprints and
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.html. Correspondence and requests for materials should be addressed to J.E.B.
ONLINE METHODS

E. coli host strains. RF0 IodoY\textsuperscript{14} was constructed from BL21(DE3) E. coli (Invitrogen) by creating a chloramphenicol-resistant F plasmid containing several key E. coli genes with recoded stop codons, knocking out RF1 in the chromosome and adding a second plasmid containing a modified Methanococcus jannaschii tyrosyl-tRNA synthetase and UAG-decoding tRNA for specifically incorporating 3-iodotyrosine at the amber stop codon\textsuperscript{15}. Cultures of RF0 IodoY were supplemented with 20 \(\mu\)g/mL chloramphenicol and 10 \(\mu\)g/mL gentamycin. RF0 Tyr was generated by adding a plasmid created by replacing the gentamycin resistance marker in the RF0 IodoY aars-tRNA plasmid with a spectinomycin resistance gene and by replacing the 3-iodotyrosine aars with the wild-type M. jannaschii tyrosyl-tRNA synthetase (GenBank accession code NP_247363.1). RF0 Tyr cultures were supplemented with 60 \(\mu\)g/mL spectinomycin and 10 \(\mu\)g/mL gentamycin. All of these strains contain a copy of T7 RNAp in the E. coli host genome under control of the lac UV5 promoter. This promoter was not induced during any of our experiments, and background expression of this host copy of T7 RNAp was apparently low enough that phages with an amber stop codon in their copy of the T7 RNAp gene could not plaque on BL21(DE3) without amber suppression.

Phage evolution. Wild-type T7 and hypermutator \(\Delta 2\) phage ancestors were obtained from I.J. Molinema (University of Texas at Austin). A single plaque of each was isolated on BL21(DE3) cells. Frozen aliquots of RF0 IodoY cells were thawed; inoculated into 50-mL Erlenmeyer flasks containing 10 mL Lysogeny Broth (LB), appropriate antibiotics and 250 \(\mu\)M 3-iodotyrosine (Sigma-Aldrich); and grown to mid-exponential phase (\(OD_{600} \approx 0.5\)) at 27 °C with orbital shaking at 120 r.p.m. Six lines of each wild-type and mutator phages were initiated by seeding separate E. coli cultures with 1 \(\mu\)L of clonal phage lysate. Phages were allowed to replicate until all of the flasks showed visible lysis by clearing of their turbidity, a period of time that varied from ~100–120 min at the beginning of the evolution experiment to ~35–45 min at the end. After lysis, 1 \(\mu\)L of each independent culture was transferred to fresh RF0 IodoY cells that had been revived from the same freezer stock and grown to mid-exponential phase. This transfer volume yields a low initial multiplicity of infection (MOI) of ~0.05–0.1. Evolved phage populations were periodically archived at ~80 °C in LB with 15% (v/v) glycerol added.

Genome sequencing and analysis. Phage DNA from each line was isolated using a CsCl gradient and sequenced using standard methods on an Illumina MiSeq instrument at the Genome Sequencing and Analysis Facility (University of Texas at Austin). Adaptor sequences were trimmed from the resulting 250-base paired-end reads using PLEXBAR\textsuperscript{20} (version 2.32).

We created updated reference sequences for the ancestral T7 wild-type and \(\Delta 2\) hypermutator phages by comparing the reads from each sample to the published T7 genome sequence\textsuperscript{12} (GenBank NC_001604.1) using bresq\textsubscript{E} (version 0.23; http://barricklab.org/bresq\textsubscript{E}) in consensus mode. The wild-type ancestor had only a single difference from the published T7 sequence: an insertion of a single A base after position 1895 in gene 0.6B. The \(\Delta 2\) ancestor had 112 genetic differences relative to the wild-type ancestor. These differences included 107 base substitutions, two single-nucleotide insertions, two single-nucleotide deletions and the in-frame 6-bp deletion in the exonuclease domain of T7 DNA polymerase that results in the hypermutator phenotype\textsuperscript{16}. Complete lists of the genetic differences between the \(\Delta 2\) and WT ancestors and the published T7 genome are available (Supplementary Data Set 1).

Mutations were predicted from the metagenomic sequencing data for the evolved populations using bresq\textsubscript{E} in two stages. The first stage used bresq\textsubscript{E} with both polymorphism and junction prediction enabled to predict large-scale structural variants. From these results, we estimated the fraction of evolved populations containing large deletions by manually examining regions with anomalous read coverage and by counting reads that mapped across new and ancestral junction sequences. The second analysis stage used bresq\textsubscript{E} in polymorphism prediction mode with no prediction of new sequence junctions. This output was used to predict point mutation variants with frequencies \(\geq 1\%\) in each population (Supplementary Data Set 1). Calculated dN/dS ratios accounted for codon abundance and assumed an equal probability of all base substitutions.

**Evolved phage population titers.** Frozen stocks were first thawed, diluted and spotted on RF0 IodoY to determine the approximate titer of each stock. Then, 100 \(\mu\)L of an appropriate dilution was added to 3 mL of melted soft agar supplemented with appropriate antibiotics and also 250 \(\mu\)M 3-iodotyrosine for RF0 IodoY. Three hundred microliters of E. coli culture at an optical density of \(\sim 1.0\) was immediately added, and soft agar was poured across the surface of LB agar plates. Plates were incubated at 37 °C for 5 h, at which time the number of plaques on each host was counted.

**Patching assays.** Phage dilutions were plated on the permissive RF0 IodoY host at 37 °C and stored at 4 °C overnight after clearly discernable plaques were obtained. The next day, E. coli host strains to be tested were inoculated into soft agar containing appropriate supplements and poured over LB plates. Isolated plaques were then picked with a pipette tip and resuspended in 1 mL of LB, and 1 \(\mu\)L of this dilution was spotted on each host strain. Plates were incubated at 37 °C, and growth was assessed after 5 h. Lysates of a collection of plaques with and without the amber suppressor–dependent phenotype were created, and the allelic state of the amber-containing gene in each phage isolate was determined via PCR and Sanger sequencing.

**Molecular modeling.** PyMOL (Version 1.5.0.4; Schrödinger, LLC) with the SwissSideChain plugin\textsuperscript{17} was used to model and visualize the 3-iodotyrosine substitution in the structure of RNA polymerase (Protein Data Bank code 1QLN).\textsuperscript{20}

**Lysis assays.** RF0 IodoY and RF0 Tyr cells were revived and grown with 250 \(\mu\)M 3-iodotyrosine and appropriate antibiotics. When cultures reached an \(OD_{600}\) of ~0.5, 200-\(\mu\)L aliquots were transferred into a clear-bottom 96-well plate and lysates of individual phage stocks, which had been previously titered on RF0 IodoY cells, were added to each well at a dilution that gave an MOI of ~5. These phages had known allelic states of the gene 17.5 amber mutation, verified by patching and sequencing. The microplate was incubated at 37 °C with \(OD_{600}\) readings taken every minute, preceded by 10 s of shaking at intensity 4, in a PowerWave 340 microplate spectrophotometer (BioTek). Lysis curves were corrected for the time it took to aliquot each separate phage and to begin making measurements in the plate reader (<10 min).

**Amber rescue mutants.** Lysate was created from an evolved WT L6 phage clone that was isolated and confirmed to have the amber mutation in the type II holin gene by patching, PCR and Sanger sequencing, as above. For most rescue mutants, multiple independent cultures of RF0 IodoY were inoculated with ~10 phage from this stock and again allowed to lyse completely, and 10\textsuperscript{-10}\textsuperscript{5} dilutions were plated on BL21(DE3) cells in soft agar as above. Other rescue mutants were isolated by plating from this stock directly on BL21(DE3) cells. In each case, mutations of the amber to nontermination codons were identified as the fastest-forming plaques, inoculated into RF0 IodoY cells to obtain lysate and genotyped via PCR and Sanger sequencing.

**Competitions.** Phages to be competed were revived from frozen stocks through two serial transfers in RF0 IodoY cells under the same conditions as the evolution experiment. After complete lysis of the second cultures, equal volumes of each phage were mixed and inoculated into new RF0 IodoY cultures to initiate competitions at 0.01×, 0.1×, 1× or 10× the transfer dilution volume used in the evolution experiment to test the effect of different initial phage MOI. Competitions proceeded for three serial transfers at their respective MOI. A sample of phage lysate was saved at the end of each infection cycle. After passing was complete, whole-phage PCR was performed with 1 \(\mu\)L of the mixed lysate as template for the region of interest. Changes in the heights of peaks corresponding to each allele in Sanger sequencing traces for these PCR products over multiple passages were used to infer the more-fit phage genotype.

**GFP emission assay.** RF0 IodoY cells were transformed with pET21 plasmids containing sfGFP variants under the control of the lac operator and a strong T7 promoter, pET21 sfGFP (WT) contained a version of superfolder GFP (sfGFP) as a positive control. pET21 sfGFP (Y66→TAG) contained an sfGFP variant with an amber codon mutation replacing Y66 in the fluorophore. Individual transformants were isolated and grown overnight with appropriate antibiotics and nCA supplements. The next day, saturated cultures were diluted 100-fold into the same medium and allowed to reach mid-log phase, at which time cultures were induced with 500 \(\mu\)M IPTG. Samples were taken 150 min later and analyzed on a Tecan Safire plate reader. An emission scan with excitation at 480 nm was performed to identify the peak emission wavelength for each.

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dissociation (CID), with up to 20 fragment spectra (MS2) collected per MS1. Dynamic exclusion was activated, with a 45-s exclusion time for ions selected more than twice in a 30-s window.

Spectra were analyzed by SEQUEST (Proteome Discoverer 1.4, Thermo Scientific) using a protein sequence database consisting of the E. coli and T7 bacteriophage proteomes, including alternative extended sequences resulting from amber readthrough. These protein sequences were generated from GenBank records NC_012971.2 and NC_001604.1, respectively. The amber codon substitution in the type II holin protein was also included for phage T7. Amber sites were designated as residue X in the sequence database, with X alternatively representing 3-iodotyrosine, tyrosine, phenylalanine or tryptophan in independent searches. The search specified full-proteolytic peptides, allowing up to two missed cleavages. Mass tolerance was set to 10 p.p.m. for precursor and 0.5 Da fragment spectra. Carbamidomethylation of cysteine (iodoacetamide) was selected as a static modification, and oxidized methionine was selected as a dynamic modification. High-confidence peptide-spectrum matches (PSMs) were filtered at <1% FDR (determined by Percolator, Proteome Discoverer v1.3, Thermo Scientific).

For the RF0 IodoY E. coli, RF0 IodoY E. coli cells lysed by the T7 phage from population WT L6 and BL21(DE3) E. coli cells lysed by wild-type T7 samples, we identified a total of 183,363, 18,527 and 20,639 PSMs, respectively. Full details of predicted PSMs that were informative about the specificity of amber readthrough are provided (Supplementary Fig. 2 and Supplementary Data Set 2).

Peptides resulting from both in-gel and in-solution digestions were desalted on Hypersep C18 spin columns (Thermo Scientific) and analyzed by nano LC-MS/MS on a Dionex Ultimate 3000 nanoRSLC system coupled to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Scientific). Data-dependent ion selection was activated with parent ion scans (MS1) collected at 100,000 (FWHM) resolution. Ions with charge > +1 were selected for collision-induced dissociation (CID), with up to 20 fragment spectra (MS2) collected per MS1. Dynamic exclusion was activated, with a 45-s exclusion time for ions selected more than twice in a 30-s window.

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