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The genetic code constrains yet facilitates Darwinian evolution

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ABSTRACT

An important goal of evolutionary biology is to understand the constraints that shape the dynamics and outcomes of evolution. Here, we address the extent to which the structure of the standard genetic code constrains evolution by analyzing adaptive mutations of the antibiotic resistance gene *TEM-1* β-lactamase and the fitness distribution of codon substitutions in two influenza hemagglutinin inhibitor genes. We find that the architecture of the genetic code significantly constrains the adaptive exploration of sequence space. However, the constraints endow the code with two advantages: the ability to restrict access to amino acid mutations with a strong negative effect and, most remarkably, the ability to enrich for adaptive mutations. Our findings support the hypothesis that the standard genetic code was shaped by selective pressure to minimize the deleterious effects of mutation yet facilitate the evolution of proteins through imposing an adaptive mutation bias.

INTRODUCTION

The genetic code plays a central role in evolutionary processes defining the relationship between DNA and protein sequences. The genetic code limits the mutational exploration of sequence space (1), as single-base changes in codons can access only about six of the 19 possible amino acid substitutions and simultaneous multiple-base changes in a codon are rare (2). Furthermore, the genetic code is biased toward conservative amino acid mutations (3). As a result, most mutational trajectories have a low probability, and probable mutational trajectories tend to be conservative in nature. Thus, very similar genes may follow different evolutionary trajectories in part because the genes' mutational neighborhoods are different (4–6) (i.e. the likely amino acid substitutions are different owing to the two genes having different but synonymous codons). What has not been experimentally addressed is the extent to which the evolution of a single gene is constrained or facilitated by the architecture of the genetic code. For a particular evolutionary outcome, how many superior fitness peaks are nearby that could have been reached if only the genetic code was arranged differently?

To the extent that the genetic code restricts a gene from evolving to higher fitness peaks, one may wonder about the possible benefits of alternative codes. However, the standard genetic code's organization makes it apparent that the relationship between DNA triplets and amino acids was not arrived at randomly. The code's arrangement has been proposed to result from the inherent interactions between amino acids and their cognate nucleotide triplets (7), the biochemical pathways through which amino acids are synthesized (8), selective pressure to minimize the deleterious effects of mutations and mistranslations (3,9) and the difficulty in changing the code once it is established (10). These basic theories have been further developed primarily through theoretical and simulation approaches (11). However, mutational bias, such as that arising from how the code is arranged or the nature of spontaneous mutations, may shape evolution (12,13). Thus, the architecture of the genetic code may facilitate the evolution of genes and proteins (14). For example, a code arranged to make adaptive mutations more likely would have provided an adaptive advantage over one that did not early in evolution when there may have been competing genetic codes. If so, the standard genetic code might still exhibit this property today. Does the standard genetic code enrich for adaptive mutations?

Here we experimentally address the extent to which the genetic code restricts access to beneficial alleles and whether the code's constraints provide advantages for the evolution of proteins. We find that although the code's architecture significantly limits evolutionary outcomes, it minimizes the deleterious cost of mutation and enriches for beneficial mutations—two properties that facilitate evolution.

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MATERIALS AND METHODS

TEM-1 β-lactamase libraries and constructs

All libraries and variants of the *TEM-1* gene were created using PFunkel mutagenesis as previously described (15).

Library selections

All antibiotics and chemical reagents for selections and MIC assays were obtained from Sigma-Aldrich. For the selection of alleles conferring cefotaxime resistance equivalent to that of *GKTS*, a previously described library (15) in which the codons for A42, E104, M182 and G238 were randomized (NNN) was plated on LB-agar plates containing 50 μg/ml spectinomycin, 50 μM IPTG and 8 μg/ml or 16 μg/ml cefotaxime. The library was plated at a cell density of 1900 or 19 000 CFU/cm^2 on the 8 μg/ml cefotaxime plates and 190 000 CFU/cm^2 on the 16 μg/ml cefotaxime plate. Plates were incubated at 37°C for 17 h. Forty colonies of ~1000 that grew on the 8 μg/ml plate and the largest 10 colonies on the 16 μg/ml plate were selected for individual screening by plate MIC assay. For colonies that passed the screen, plasmid DNA was isolated from overnight cultures using the Qiagen QIAprep Spin Miniprep kit (27106) and the *TEM-1* allele was sequenced. Unique plasmids were retransformed into fresh DH5α *E. coli* cells, and the MIC determined by both plate and liquid MIC assays.

For the selection of *TEM-1* alleles with a single amino acid substitution that provides increased resistance to cefotaxime, two previously described *TEM-1* comprehensive codon libraries were used: CCM1 and CCM2 (15). DH5α cells bearing CCM1 were plated at a density of 100–300 CFU/cm^2 on LB-agar plates containing 50 μg/ml spectinomycin, 50 μM IPTG and 0.04 μg/ml cefotaxime. NEB 5-alpha F'I^q cells bearing CCM2 were plated as above except the cell density was 150–600 CFU/cm^2, the cefotaxime was 0.02 μg/ml and the IPTG was 300 μM. The concentrations of cefotaxime used correspond to the MIC conferred by *TEM-1* in DH5α and NEB 5-alpha F'I^q cells. Plates were incubated at 37°C for 17 h. The *TEM-1* gene of randomly selected colonies was sequenced. Because any particular amino acid substitution is relatively rare in the libraries, we used the criteria that an amino acid substitution had to be observed twice for us to categorize it as adaptive.

MIC assays

For MIC assay on agar plates, cultures of variants were prepared in LB broth at 37°C with shaking at 250 rpm until all cultures reached saturation, ~24 h. Cultures were diluted 100-fold in LB broth, and incubated for ~2.5 h until the OD was about 0.3. The cultures were diluted to 10^4 CFU/μl, and 1 μl was spotted on Mueller–Hinton agar plates containing 50 μg/ml spectinomycin, 50 μM IPTG and $\sqrt{2}$-fold increasing concentrations of cefotaxime. The plates were incubated at 35°C for 20 h. The MIC was determined as the minimal concentration at which no growth was observed.

For liquid MIC assays, the initial cultures were prepared as above and then diluted to a concentration of 1×10^6 CFU/μl in Mueller–Hinton broth. A total of 150 μl of this diluted culture was added to wells of a 96-well assay plate along with 150 μl of Mueller–Hinton broth containing 100 μg/ml spectinomycin, 100 μM IPTG and 2-fold increasing concentrations of cefotaxime. The plate was covered and sealed in a plastic bag and incubated at 35°C for 20 h. The MIC was determined as the minimal concentration at which no visible growth was observed.

The above two MIC tests used different temperatures and media than the selections. The MIC assay conditions used are standardized conditions for quantifying beta-lactam resistance that allow comparisons with other studies (16).

Enrichment values of experimentally observed codon substitutions

Enrichment values for each codon substitution introduced in *HB36.4* and *HB80.3* were determined by using custom Matlab scripts to analyze the Illumina deep sequencing data on the libraries before and after selection for hemagluttinin binding. The data were filtered as in the study by Whitehead *et al.* (17) to include sequencing reads with only one codon substitution, and the final list to include only codon substitutions with at least 100 sequencing counts in the reference library. The enrichment value E was calculated as

$$E = \log_2 \left(\frac{(\textit{selected library counts}) / (\textit{total selected library counts})}{(\textit{reference library counts}) / (\textit{total reference library counts})} \right) \quad (1)$$

Thus, E quantifies the relative prevalence of an allele in the selected library compared with the reference library (the naïve library). The enrichment value of the wild-type sequence was determined by averaging the enrichment values of all codons synonymous with the wild type. Codon substitution counts, enrichment values and the wild-type enrichment values were consistent with values for amino acid substitutions presented by Whitehead *et al.* (17).

The genetic code's enrichment of adaptive mutations and meta-analysis

The percent enrichment and *P*-values were determined as described in Supplementary Table S6. Meta-analysis on the *P*-values was performed using the Stouffer's Z-trend method (weighted Z score) using the program MetaP (http://people.genome.duke.edu/~dg48/metap.php).

RESULTS

The natural and *in vitro* evolution of TEM-1 β-lactamase for conferring cefotaxime resistance converges on the same set of mutations

We chose to examine the genetic code's constraints on evolution with the antibiotic resistance *TEM-1* gene encoding TEM-1 β-lactamase—a gene that has provided many

insights into how epistasis constrains evolution (16,18–20). TEM-1 hydrolytically inactivates β-lactam drugs such as penicillin, but has very low activity on the third-generation cephalosporin β-lactam cefotaxime. Clinically isolated alleles of *TEM-1* conferring elevated antibiotic resistance arise through accumulation of point mutations (i.e. 1-bp substitutions). For example, *TEM-52* differs from *TEM-1* by three point mutations resulting in the E104K/M182T/G238S mutations (21) that increase cefotaxime resistance ~4000-fold (16). The *in vitro* evolution of *TEM-1* mimics its natural evolution (22). Six independent *in vitro* evolution studies that applied selective pressure for increased cefotaxime resistance found the E104K/M182T/G238S combination of mutations in the best alleles (20,22–26). A fourth mutation (A42G) (25) that also arises from a point mutation increases cefotaxime resistance to about 33 000-fold over *TEM-1* (16). The high fitness of the gene bearing the A42G/E104K/M182T/G238S mutations (referred to here as *GKTS*) has not been surpassed by increasing the mutation rate (20,26), using mutator strains of bacteria (24), forcing explorations of alternative trajectories through use of bottlenecks (20), or using computational protein optimization strategies (which are not constrained by the genetic code) (27). This suggests that the evolutionary outcome of *GKTS* is largely reproducible and inevitable, given a strong selective pressure for cefotaxime resistance (16). Among the accessible local optima for cefotaxime resistance on the β-lactamase fitness landscape, *GKTS* may be the global optimum.

To what extent did the architecture of the genetic code direct this outcome? There are $20^4 - 1 = 159\,999$ possible amino acid combinations at these four positions in TEM-1 (including combinations with up to three wild-type amino acids). However, only 2743 (i.e. $7 \times 8 \times 7 \times 7 - 1$) or 1.7% of these are readily accessible combinations, as they do not require simultaneous multiple mutations in any one codon, which is a rare occurrence. Synonymous mutations followed by a second point mutation can expand the accessible amino acid combinations, but only to some extent. Also, subsequent second mutations in codons with previously accumulated beneficial mutations can occur, but this requires that both mutations be beneficial and that the second mutation increases the fitness of the gene. This significant constraint will keep the readily accessible combination of mutations at these four codons low. More to the point, such double mutations are not present in the best cefotaxime resistance alleles arrived at by natural or *in vitro* evolution of *TEM-1*. The rarity of natural adaptive mutations with multiple-base substitutions in a single codon is exemplified by a recent study that examined 516 spontaneous ceftazidime-resistant isolates of *Burkholderia thailandensis* and found 29 different codon substitutions in the *penA* β-lactamase gene that provided this resistance—all of which were point mutations (28). In addition, the occurrence of reciprocal sign epistasis will further constrain which combination of amino acids are accessible by evolution (29).

The genetic code constrains the evolution of *TEM-1*

To test the extent to which the genetic code constrains the evolution of *TEM-1*, we asked whether there exist other amino acid combinations at these four positions that provide fitness equal to or better than *GKTS*. We used a library in which these four codons were randomized at all three base positions (15). We placed the mutated gene downstream from the IPTG-inducible *tac* promoter on a plasmid with the *p15A* origin (copy number ~10), as in previous *in vitro* evolution experiments with *TEM-1* (20,22). Our library consisted of 5.8 million transformants (short of the theoretical $4^{12} = 16.8$ million DNA variants, but in excess of the possible $20^4 = 160\,000$ protein variants), and the majority of library members contained mutations at all four positions (15). Although we subsequently determined in separate experiments that the degenerate oligos used to make the library were enriched for G's by about 2.2-fold (15), a large fraction of the protein variants are likely to be present in the library.

We subjected this library to selections for cefotaxime resistance equivalent or superior to that achieved by *GKTS*. We performed a secondary screen on 50 of the resulting ~1000 colonies for those with a MIC at or above that conferred by *GKTS*. Clones passing this screen were sequenced. The plasmid DNA from unique clones was retransformed into fresh DH5α *E. coli* and the cefotaxime MIC determined by solid and liquid media growth assays.

The sequences and corresponding MICs revealed that there are many alleles with equivalent or superior combinations of amino acids at these four positions (Table 1). Of the 17 identified alleles (11 unique amino acid combinations), only four were identified more than once, indicating that there are additional high fitness alleles yet to be found. Although we observe small differences (up to two-fold) between synonymous alleles in the plate MIC assay (Table 1), this assay showed variability in replicate experiments of up to two-fold in some instances (Supplementary Table S1). Six unique amino acid sequences differed from GKTS at two of the four positions. Most strikingly, 55% (6 of 11) of the amino acid combinations identified require more than one point mutation in at least one codon (i.e. Hamming distance >4). We find the existence of numerous equivalent or superior alleles nearby what appeared to be a dominant resistance allele quite striking. This experiment shows that there are many alleles equivalent or superior to *GKTS* nearby in sequence space that are not readily accessed by natural or *in vitro* evolution

To address whether there is a mutational pathway to any of the alleles with a Hamming distance >4, we chose *GKQA* (codons: ggg-aag-cag-gca) as a representative allele and constructed the 14 combinations of these four codon substitutions. We considered each codon substitution (whether a point mutation or a multi-bp substitution) as a single mutational step in order to ask whether *GKQA* could be reached if the genetic code were arranged differently such that each of the required amino acid substitutions were possible with a point mutation. We tested the cefotaxime resistance of these variants and assessed the feasibility of the 24 possible trajectories from *TEM-1* to *GKQA*. We assumed that the evolution of *TEM-1* fits the strong selection/weak mutation model of evolution by which the time to fixation or loss of a mutation is much

Table 1. Cefotaxime resistance of selected TEM-1 β-lactamase alleles

Colony[a]	For positions 42-104-182-238		Number of base changes in each codon[c]	MIC (μg/ml)[d]	H (actual)[e]	H (minimum)[f]
	Amino acids[b]	Codons				
	No TEM-1 gene			0.08		
TEM-1	A-E-M-G	gca-gag-atg-ggt	0-0-0-0	0.08	0	0
GKTS	G-K-T-S	gga-aag-acg-agt	1-1-1-1	90.5	4	4
43, 48	G-K-**M-A**	ggg-aag-atg-gcg	2-1-0-2	90.5	5	3
24	G-K-**M**-S	ggg-aag-atg-tca	2-1-0-3	45.3	6	3
2	G-K-**K-A**	ggg-aag-aag-gct	2-1-1-1	64	5	4
6	G-K-T-**A**	gga-aag-acg-gct	1-1-1-1	90.5	4	4
34		ggg-aag-acg-gcg	2-1-1-2	181	6	4
9		ggg-aag-aca-gcc	2-1-2-2	181	7	4
32	G-K-T-S	ggg-aag-acg-tcg	2-1-1-3	90.5	7	4
1	G-K-**A-A**	ggg-aag-gcg-gct	2-1-2-1	90.5	6	5
38	G-K-**A**-S	ggg-aag-gcg-agc	2-1-2-2	90.5	7	5
16		ggg-aag-gcc-agc	2-1-3-2	64	8	5
14	G-K-**Q-A**	ggg-aag-cag-gca	2-1-2-2	128	7	5
5, 31		ggg-aag-cag-gcc	2-1-2-2	90.5	7	5
7, 15		ggc-aag-caa-gca	2-1-3-2	64	8	5
46	G-K-**S-A**	ggg-aag-agc-gct	2-1-2-1	128	6	5
33	G-K-**S-S**	ggg-aaa-agt-agt	2-2-2-1	90.5	7	5
3	G-**R-S-S**	ggg-cgg-agc-tcg	2-2-2-3	64	9	6
45, 49		ggt-aga-tct-tcg	2-3-3-3	128	11	6

[a]Two numbers indicate that the allele was found twice.
[b]Bold indicates amino acids differing from those in GKTS.
[c]Relative to *TEM-1*.
[d]Median value of three replicates. Assays performed in $\sqrt{2}$ increments of cefotaxime (Mueller–Hinton-agar, 10^4 CFU/spot, 35°C for 20 h). Data for all replicates are in Supplementary Table S1. MICs determined by Mueller–Hinton broth liquid growth assay at 35°C can be found in Supplementary Table S2.
[e]Hamming distance between the allele and *TEM-1*.
[f]Minimum Hamming distance to achieve same amino acid sequence.

shorter than the time between mutations. Thus, we required that mutations accumulate one at a time with increasing fitness at each step for a trajectory to be deemed feasible, as in a previous study (16). Nine of the 24 possible trajectories were feasible (Figure 1). Four trajectories ended at an intermediate (*GKMA*) with equivalent resistance to *GKQA*. Like the feasible trajectories for evolving *GKTS* (16), the first mutation necessarily occurs at positions 104 or 238, and the fittest double mutant has mutations at both positions (the difference is that 238 is mutated to A instead of S for *GKQA*). A lack of a mutational trajectory cannot explain why *GKQA* has not been found in the natural or *in vitro* evolution of *TEM-1*. Instead, we posit that the requirement for multiple mutations in a single codon is one reason that makes this allele's occurrence unlikely. Thus, the architecture of the genetic code constrains evolution by making some viable mutational trajectories improbable.

Epistasis and mutational bias also constrain the exploration of sequence space

Alleles such as *GKMA* and *GKTA* provide resistance equivalent to *GKTS* (Table 1) and have amino acid combinations that can be reached by three or four point mutations, respectively, yet these combinations have not been previously identified in laboratory evolution experiments. We speculate that there are two reasons why such these alleles have not been previously identified. The first reason is epistasis. For example, G238A was more common in the identified alleles than G238S and is present in both *GKMA* and *GKTA*. However, when occurring as the only mutation in TEM-1, G238S provides a 4-fold higher k_{cat}/K_m and a 4-fold higher MIC than G238A (30). Because G238S is the amino acid substitution that, by itself, increases cefotaxime resistance the most, it is most likely to be fixed first. The apparent equivalence of G238A and G238S among our selected alleles is illustrative of the epistatic nature of mutations. The second reason is mutational bias. For example, the G:C → C:G mutation necessary for G238A is considerably less common than the G:C → A:T mutation for G238S in error-prone PCR reactions (31) and spontaneously in *E. coli* (32).

The genetic code minimizes the fitness cost of mutations

There is no arrangement of a 20 amino acid / 64 codon genetic code that would not significantly limit the types of

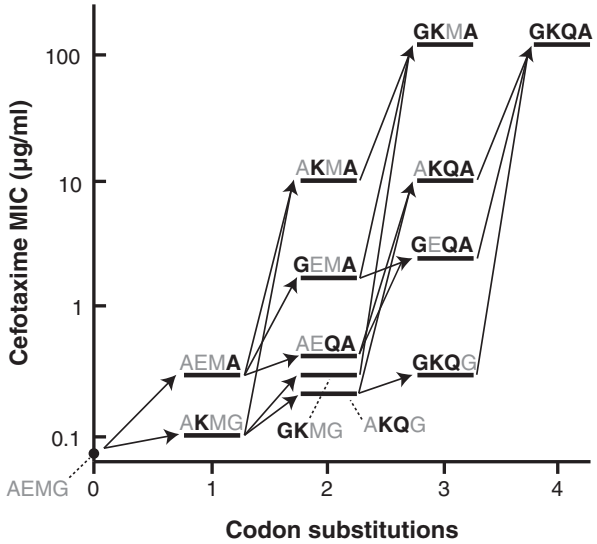


Figure 1. Feasible trajectories for evolving *GKQA* (colony 14) from *TEM-1* (i.e. *AEMG*) by accumulation of codon substitutions one at a time. Mutations are shown in black, bold letters. Of the 24 possible trajectories, five end with *GKQA* and four end with *GKMA*, an allele with equivalent fitness to *GKQA*. Cefotaxime resistance was measured by plate assay as in Table 1, and the value reported represents the median of three replicates. Data for all replicates are provided in Supplementary Table S3.

amino acid substitutions that are readily accessible, as a single codon can be mutated only to nine other codons with a point mutation. Thus, different genetic codes will constrain evolutionary paths in different ways. The adaptive theory of the origin of the genetic code postulates that the code's conservative architecture is a result of selective pressure to minimize the deleterious effects of point mutations and mistranslation errors (3,9). The adaptive theory predicts that for non-synonymous mutations, the average fitness cost of point mutations should be less than that of 2-bp and 3-bp substitutions. However, such a test of the adaptive theory (and of the conservative nature of the code) has never been systematically applied to any gene. How does the mutational fitness distribution partition between 1-, 2- and 3-bp codon substitutions of a gene?

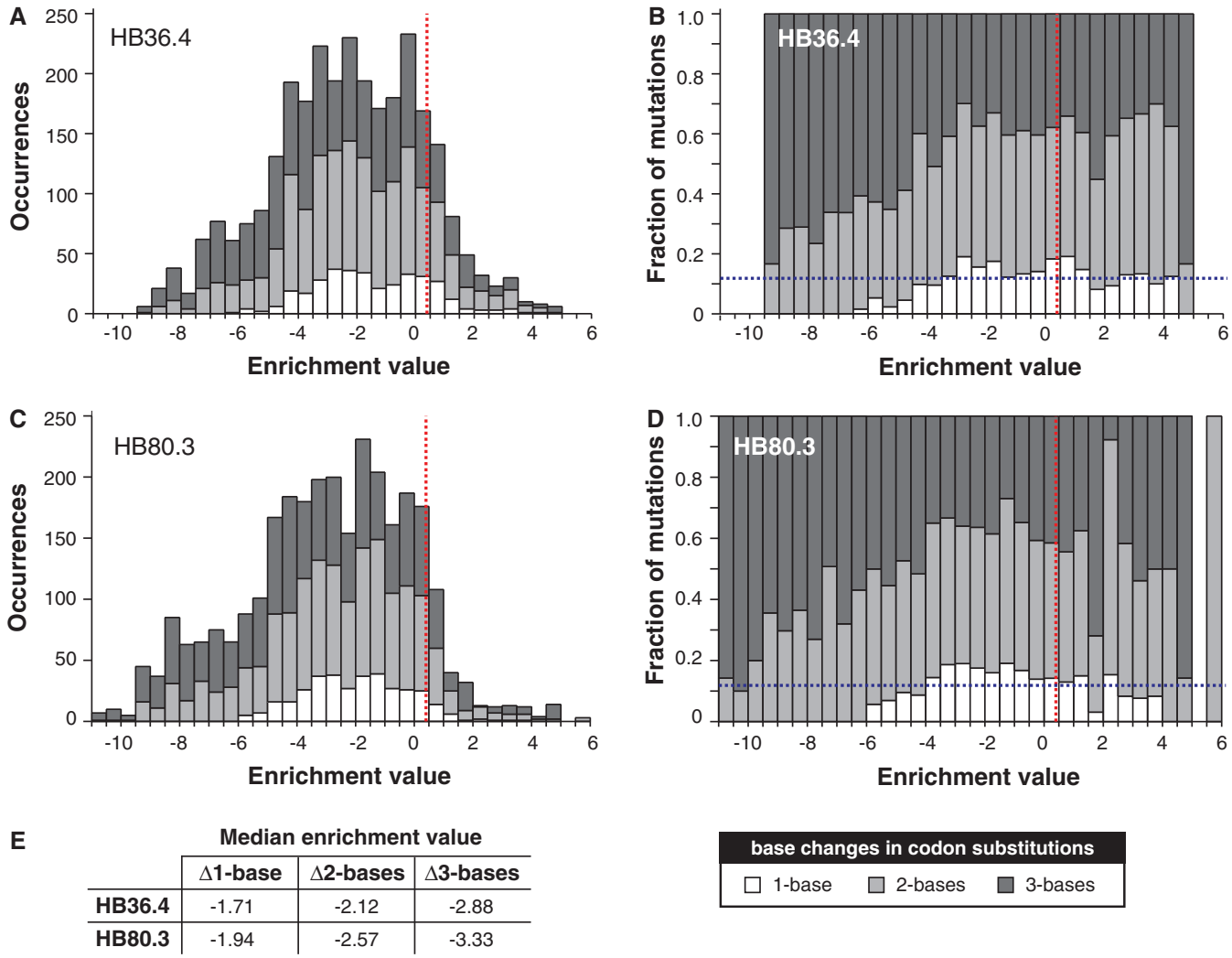
To address this question, we examined the distribution of fitness effects of 1896 unique single amino acid substitutions in two genes that were previously modified through a combination of computational design and directed evolution to inhibit H1N1 influenza hemagglutinin (17,33). Inhibitor HB36.4 derives from Apc36109 from *Bacillus stearothermophilus* and HB80.3 from the Myb domain of the Rad transcription factor from *Antirrhinum majus* (33). Because the natural proteins are not inhibitors of hemagglutinin, we suggest that HB36.4 and HB80.3 should be viewed as genes that are not evolutionarily mature for hemagglutinin inhibition. Whitehead *et al.* (17) created NNK degenerate codon libraries consisting of all possible single amino acid substitutions in all 51 positions in HB80.3 and 53 of 93 positions of HB36.4. In NNK libraries, the first two nucleotides in a codon can be any base, but the third nucleotide is limited to G or T to reduce the frequency of nonsense codons while still allowing all possible amino acids. The libraries were subjected to deep sequencing before and after selection for hemagglutinin binding in a yeast display format. In their study, the base 2 logarithm of the ratio of the frequencies of each amino acid substitution in the selected versus unselected libraries—referred to as the enrichment value—served as a proxy for the change in free energy of binding. Several lines of evidence support the suitability of this proxy (17).

Because differences in enrichment values for synonymous mutations were considerably smaller than differences between non-synonymous mutations (17), we assigned the enrichment values of each amino acid substitution to its respective codon substitutions and used this value as a proxy for the change in gene fitness caused by the 5857 codon substitutions (i.e. the number of unique codon substitutions that can code for the 1896 unique amino acid substitutions observed). The distribution of these fitness effects (Figure 2A–D) indicates that the standard genetic code's architecture asymmetrically partitions fitness effects of amino acid substitutions between point mutations and multi-bp codon substitutions and minimizes the fitness cost of point mutations. The average cost of mutation for point mutations is substantially less than for 2-bp substitutions, and 3-bp substitutions have the highest average fitness cost (Figure 2E). Mutations causing a smaller decrease in fitness are enriched in point mutations, and mutations with the largest negative effect are almost exclusively 2- and 3-bp changes. The same distribution trends are observed when we determined the enrichment values for the 2813 experimentally observed codon substitutions (Supplementary Figure S1). Among beneficial mutations, the median effect of mutations for multi-bp mutations was marginally higher than that of point mutations (Supplementary Table S4).

We interpret these results (Figure 2E) as evidence that the code's arrangement minimizes the fitness cost of amino acid substitutions. An alternative explanation is that the genes are products of evolution under the standard genetic code, and thus their make-up is such that point mutations will cause minimal deleterious effects. However, this viewpoint is mitigated somewhat by the fact that HB36.4 and HB80.3 were not evolved by nature for hemagglutinin binding, but rather are a product of codon optimization for yeast expression, computational design and limited laboratory evolution. The genes may better represent ones in the process of evolving rather than 'evolutionarily mature' genes.

The genetic code is biased towards adaptive mutations

Figure 2 shows that readily accessible amino acid substitutions (i.e. those from point mutations) have smaller deleterious effects on fitness. How does this affect the evolution of proteins? For a gene with L codons, there are $19L$ possible amino acid substitutions. The standard genetic code imposes a restriction on which of these $19L$ are likely (i.e. on average, only about $6L$ occur with a point mutation; the exact number for a particular gene will depend on the gene's DNA sequence). In other words, the genetic code provides a set of codon-based



	Δ1-base	Δ2-bases	Δ3-bases
HB36.4	-1.71	-2.12	-2.88
HB80.3	-1.94	-2.57	-3.33

Figure 2. Distribution of fitness effects of non-synonymous codon substitutions in (**A** and **B**) *HB36.4* and (**C** and **D**) *HB80.3*. The distribution is partitioned into codon changes with 1-, 2- and 3-base changes. The red dashed vertical line indicates the enrichment value of the parental genes, and the blue dashed horizontal bar indicates the fraction of all possible mutations of the gene that are point mutations. Enrichment values for parental genes are slightly greater than zero because most mutations have a negative effect on fitness. (**E**) Median enrichment values for types of codon substitutions. Distributions based on codon enrichment values instead of amino acid enrichment values are provided in Supplementary Figure S1.

rules governing which amino acid substitutions readily occur. Are these rules biased toward adaptive mutations? If so, then the code's arrangement would provide an advantage because accessible amino acid substitutions would be more likely to be adaptive than randomly chosen amino acid substitutions (Figure 3).

We examined this question in *HB36.4*, *HB80.3* and *TEM-1* by first identifying an extensive set of adaptive amino acid substitutions in these genes. For our analysis of *HB36.4* and *HB80.3*, we used the set of amino acid substitutions enriched over wild-type as determined by Whitehead *et al*. (17). For *TEM-1*, we previously constructed comprehensive codon mutagenesis libraries that consisted of ≥97% of all 18 081 possible single codon substitutions in *TEM-1* (i.e. 63 possible codons × 287 positions in *TEM-1*) (15). From these libraries, we previously identified 38 tazobactam resistance alleles (19 unique amino acid substitutions)—tazobactam being an inhibitor of TEM-1 (15). Here, in an analogous manner, we

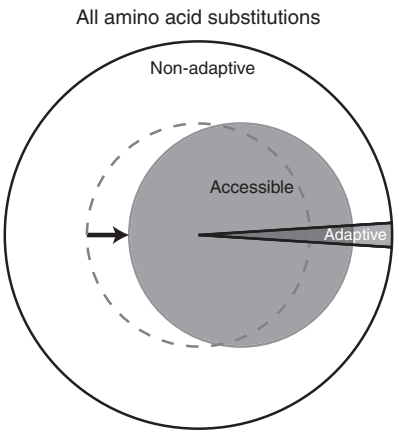


Figure 3. Enrichment of adaptive amino acid substitutions of genes by the standard genetic code. The filled gray circle depicts a code in which point mutations preferentially access adaptive amino acid substitutions while the dotted circle depicts a non-enriching code that randomly samples amino acid substitutions.

Table 2. Enrichment for adaptive mutations provided by the standard genetic code

Gene	Adaptive advantage	% Enrichment of adaptive amino acids[a]
TEM-1	Cefotaxime resistance	39.6 ($P = 0.106$)
TEM-1	Tazobactam resistance	35.6 ($P = 0.210$)
HB36.4	Hemagglutinin binding	30.6 ($P = 0.0066$)
HB80.3	Hemagglutinin binding	0.51 (not significant)

[a]Details on this calculation provided in Supplementary Table S6. The *P*-values provide the probabilities that the observed enrichment was arrived at by chance under the null hypothesis that adaptive mutations are as likely to be accessible by point mutations as non-adaptive mutations.

identified 77 cefotaxime resistance alleles (30 unique amino acid substitutions) by sequencing the *TEM-1* gene of 500 colonies that formed when the library was challenged to grow on plates with elevated levels of cefotaxime (Supplementary Table S5). Whereas the adaptive amino acid substitutions identified in *HB36.4* and *HB80.3* span the range from smallest to largest beneficial effect, those identified in *TEM-1* are the amino acid substitutions with the largest adaptive effect (see Supplementary Text).

We then calculated the fraction of adaptive amino acid mutations that could be reached with a point mutation and compared that with the fraction of all amino acid substitutions that are possible with a point mutation. For these genes, the standard genetic code enriched for adaptive amino acid substitutions up to 40% (Table 2). We assessed the significance of this result by comparing the experimentally determined number of adaptive mutations accessible by a point mutation with the distribution of that value expected if adaptive mutations were chosen at random from all possible mutations (a hypergeometric distribution). The *P*-value obtained reflects the probability of arriving at that enrichment value or higher by chance under the null hypothesis that adaptive amino acid substitutions are no more likely to be accessible by a point mutation than are all possible amino acid substitutions. The relatively small number of adaptive amino acid substitutions identified for *TEM-1* makes obtaining low *P*-values unlikely unless the enrichment is very large. If the null hypothesis was true, we would expect to find that enrichment would be found as often and to the same extent as depletion; however, depletion was not observed in any of the four sets of adaptive mutations examine. Considering the *P*-values of all four experiments collectively by meta-analysis (34), we find that the enrichment observed in our experiments is significant ($P = 0.0027$). This result supports our hypothesis that the standard genetic code, by its limitations on which amino acid substitutions are accessible by a point mutation, facilitates the evolution of proteins by enriching for adaptive mutations.

DISCUSSION

To the extent our results with these three genes can be generalized, our results indicate that the standard genetic code possesses a remarkable feature—it provides the advantage of reducing the negative effects of mutations while selectively enriching for adaptive mutations. Thus, although the code's structure limits the exploration of sequence space, it does so in a manner that benefits the evolution of proteins and is a molecular-level example of how constraints can facilitate evolution (35). We speculate that the architecture of the code results in part from selective pressure for a code that facilitates the evolution of proteins. This evolvability theory on the origin of the genetic code is not mutually exclusive with existing theories and offers additional insight into the origin of the standard genetic code.

Our use of the term 'evolvability' and our proposal that the code's enrichment for adaptive mutations provided an adaptive advantage requires further clarification. When compared with other genetic codes that do not enrich for adaptive mutations, the standard genetic code imposes a bias toward adaptive mutations in the standing genetic variation. Of course this bias would not be true for all genes or in all possible environments. Rather, we contend it would be true on average. Thus, provided that adaptation is limited by the supply of adaptive mutations, the standard genetic code would confer a higher degree of evolvability than a code that does not enrich. This advantage the code possesses invokes clade selection, as it provides an advantageous backdrop for adaptive evolution. As evolution proceeds, surviving lineages would become increasingly biased toward those with this code, which experienced more beneficial mutations sooner than their competitors.

Whether this evolvability provided an adaptive advantage (as we propose) or is a byproduct of evolution is a difficult question to answer (36,37). However, our proposal does not suffer from many criticisms of evolvability involving clade selection (37,38). First, we do not need to invoke increased mutation rates or capacity to produce new variation as the source of evolvability. Rather, we contend that the standard genetic code provides a *better* standing genetic variation for evolution than would codes that do not enrich for adaptive mutations. Thus, early in evolutionary history, this could have provided an adaptive advantage contributing to the standard genetic code winning out over alternative codes that may have been present at the time. Second, the genetic code is not a simple 'variability allele' that is prone to being lost by recombination because it is subject to indirect selection. The genetic code is a manifestation of a large set of genes and is central to life. It cannot be lost, and it is difficult to think of ways in which the code would be vulnerable to 'selfish' alternatives. Thus, although our results indicate that the standard genetic code possesses the ability to enrich for adaptive mutations today and in the future, we are not invoking a teleological view of evolution. Rather, the adaptive advantage existed earlier in evolution in the context of other competing codes while genomes were smaller and the genetic code exhibited plasticity. We believe that the code's retention of this feature is a testament to how difficult it is to substantially change the genetic code after its fixation in the last universal common ancestor (10).

Our results support the idea that both robustness to error and improved access to adaptive mutations were selected for in the genetic code's evolution. We speculate that there are two possible ways in which our evolvability theory can be reconciled with the adaptive theory vis-à-vis error minimization. (i) First, perhaps a code's error minimization must be balanced by its propensity to promote the evolution of proteins. A code maximized for robustness to error would allow only the most conservative of mutations, which may not be optimal from the perspective of protein evolution. We postulate that a code that allows for the right balance between error minimization and effective exploration of sequence space would be evolutionarily advantageous. In this view, the evolvability theory provides a possible explanation for the extent to which, if any, the code is not optimized for error minimization (11). (ii) Second, it may be that the error minimization and adaptive mutation enrichment provided by the genetic code are two sides of the same coin. Potentially, a conservative genetic code increases the probability of achieving an adaptive mutation by reducing the effect of the mutations (39), consistent with Fisher's Geometric Theorem (40). If error minimization and enrichment for adaptive mutations do come together as a package, an interesting but difficult question to address experimentally is the extent to which each contributed to the origin of the genetic code.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–6, Supplementary Figure 1, Supplementary Text and Supplementary References [15,41,42].

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