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Genetics

Membership mutation of the genetic code: Loss of fitness by tryptophan

(amino acid analogue/4-fluorotryptophan)

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ABSTRACT **_Bacillus subtilis_ strain QB928, a tryptophan-auxotroph, was serially mutated to yield strain HR15. For QB928, tryptophan functioned as a competent amino acid and 4-fluorotryptophan as merely an inferior analogue. For HR15, these roles were reversed. The tryptophan/4-fluorotryptophan growth ratio decreased by a factor of 2×10^4 in the transition from QB928 to HR15.**

The genetic code distributes 64 triplet codons to 20 amino acids and three termination signals for the construction of protein molecules. In the evolution of the code, membership in the ensemble of encoded amino acids may undergo addition, deletion, or replacement; without changing membership, codons also may be redistributed among the amino acids. Purely codonic changes are implicated in the departure of mitochondrial codes from the universal, or mainstream, code (1–3) and occur to a limited extent in missense and nonsense suppressions (4, 5).

Whether or not any membership change has ever occurred in the course of biological evolution is more difficult to decide. Constituents of polysaccharides and lipids are varied. Even with nucleic acids, the use of hydroxymethyl-dCTP as substrate for T4 bacteriophage DNA synthesis at least exemplifies a variation. However, prokaryotic, eukaryotic, mitochondrial (6), and chloroplast (7) proteins uniformly utilize the same 20 proteinous amino acids. Thus there is no evidence from extant organisms that the encoded amino acids have ever changed during three billion years of biological evolution, in spite of the selective advantage to be gained from increased variety in amino acid side chains, as witnessed by as many as 120 kinds of post-translational modifications of proteins (8), and the availability of over 200 kinds of novel amino acids from cellular metabolism (9) for entry into the code. The invariance is also largely not due to protection by the specificity of aminoacyl-tRNA synthetases; these enzymes distinguish among the 20 incumbent amino acids with extreme fidelity but they permit the incorporation of numerous amino acid analogues into proteins either partially or, in the case of selenomethionine (10) and trifluoroleucine (11), completely in place of an incumbent amino acid. Instead, what appears to be the most powerful barrier stabilizing the code has been the continual optimization of protein sequences on the basis of the incumbent amino acids, such that the replacement of any of them nowadays by an analogue typically brings a steep decline in biological fitness, as measured by cell growth (12, 13). This fitness edge has to be not only removed but also reversed in order to achieve a membership change in the code.

The coevolution theory of the genetic code proposes that the development of the universal code was inseparably linked to that of pathways for amino acid biosynthesis (14–18). New amino acids formed by primitive catalytic pathways received part or all of the codons of precursor amino acids through pretranslational modification of, or competition against, the precursors. This explains the entry of prebiotically unavailable amino acids into the code as well as the overwhelming correlation existing between codon allocation and biosynthetic precursor–product relationships. The theory requires that the genetic code went through a period of active turnover and selection of the membership amino acids, which ceased only after the highly versatile ensemble of 20 standard amino acids had become entrenched. Furthermore, because the rules of evolution remain the same now as always, the theory predicts that continued change should be possible, particularly through the competition mechanism. Accordingly, in the present study an attempt is made to resume the process of mutational change, after a lapse equal to half of earth's history, by selecting against the fitness of an incumbent amino acid relative to that of a competitive analogue.

MATERIALS AND METHODS

Growth of Bacteria. *Bacillus subtilis* strain QB928 (*aroI906 purB33 dal trpC*$_2$) was provided by J. Mandelstam. Basal medium B was similar to that employed by Sterlini and Mandelstam (19) and contained per liter 0.15 g of glutamic acid, 0.15 g of glycerol, 1.36 g of KH_2PO_4, 0.11 g of Na_2SO_4, 0.6 mg of $FeCl_3 \cdot 6H_2O$, 0.54 g of NH_4Cl, 0.096 g of NH_4NO_3, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2$, and 22 mg of $MnSO_4 \cdot 4H_2O$; the final pH of the medium was adjusted to 7.0. Medium G was made up by adding to each 100 ml of medium B 1 ml of nucleoside solution (5 mg each of guanosine, adenosine, cytidine, and uridine per ml and 25 mg of thymidine per ml), 1 ml of vitamin-amide solution (1 mg of thiamine, niacinamide, and pyridoxal per ml, 0.2 mg of pantothenic acid per ml, 0.1 mg of biotin, thioctic acid, and *p*-aminobenzoic acid per ml, 0.05 mg of folic acid and riboflavin per ml, 15 mg of ribitol per ml, and 5 mg of glutamine and asparagine per ml), 2.5 ml of 20% glucose, 2.5 ml of DL-alanine at 4 mg/ml, 2 ml of amino acid solution (1 mg each of arginine, aspartic acid, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine per ml). To support growth, unless otherwise specified, medium G was supplemented with 5 μg of either Trp or DL-4-fluorotryptophan (4-FTrp; Sigma) per ml. Cells were grown at 37°C in liquid medium or on 1.7% agar and could be stored frozen at −77°C or −196°C suspended in medium G containing 0.5 M sucrose. Growth in suspension was monitored by optical density at 450 nm. Growth on agar was measured by a modification of the phosphate method of Bennett and Williams (20); cells grown on

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Abbreviation: 4-FTrp, DL-4-fluorotryptophan.

agar containing [^{32}P]phosphate were removed by 3 ml of medium B with the aid of a glass scraper, and an aliquot was assayed for radioactivity by scintillation spectroscopy after acidification with 5% trichloroacetic acid and washing on a Millipore 0.45-μm filter.

Mutant Isolation. For mutagenesis, liquid culture in medium G with 4-FTrp was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 100 μg/ml for 20 min, washed five times in medium B, and again grown in medium G with 4-FTrp. Application of penicillin treatment entailed washing the cells at this stage free of 4-FTrp and resuspending in medium G with Trp in the presence of 2 units of penicillin per ml/0.5 M sucrose. After shaking for 3 hr, cells were washed twice with medium G.

Amino Acid Analysis. Cells grown in liquid medium G supplemented with Trp or 4-FTrp were harvested at late logarithmic phase, washed three times by centrifugation in medium B, and frozen. Upon thawing, 0.3 g of cells was suspended in 3 ml of 0.5 M NaOH at 0°C and precipitated with 3 ml of ethanol. The pellet was washed once in 1:1 mixture of 0.5 M NaOH and ethanol, three times in 50% ethanol, once in a 1:1 mixture of ethanol and ether, and finally once in ether. The dried powder was hydrolyzed with 3 M mercaptoethanesulfonic acid for 20 hr at 110°C and neutralized (21). An aliquot was chromatographed on a Beckman 121 amino acid analyzer by using a 0.9 × 18 cm column of PA35 resin (flow rate, 70 ml/hr) in 14.6 mM sodium citrate at pH 5.65. Ninhydrin color at 570 nm (18-mm pathlength) of effluent from column was recorded from 25 min onward after the passage of acidic and neutral amino acids.

Table 1. Trp/4-FTrp growth ratios

Strain	Radioactivity, cpm/ng of cells plated		Trp/4-FTrp
	Trp	4-FTrp	
QB928	170,000	230	730
LC8	230,000	630	360
LC33	90,000	52,000	1.7
HR7	21,000	32,000	0.66
HR15	1,100	31,000	0.036

Cells were grown for 46 hr on 20 ml of G agar containing 5 μg of Trp or 4-FTrp per ml and 4.2 μCi of [^{32}P]phosphate. From 2.5 to 5 ng of QB928 and LC8 (dry weight) and six times as much of the slower growing LC33, HR7, and HR15 were spread per plate.

RESULTS

Isolation of Strain LC33. The Trp-auxotrophic *B. subtilis* QB928 cells yielded no visible colonies on 5 μg of 4-FTrp per ml but yielded tiny colonies on 20 μg of 4-FTrp per ml. From a thick spread of the latter, a somewhat larger colony was picked and designated LC8. Growth of LC8 again on 20 μg of 4-FTrp per ml yielded LC33, which displayed a clear large colony char-

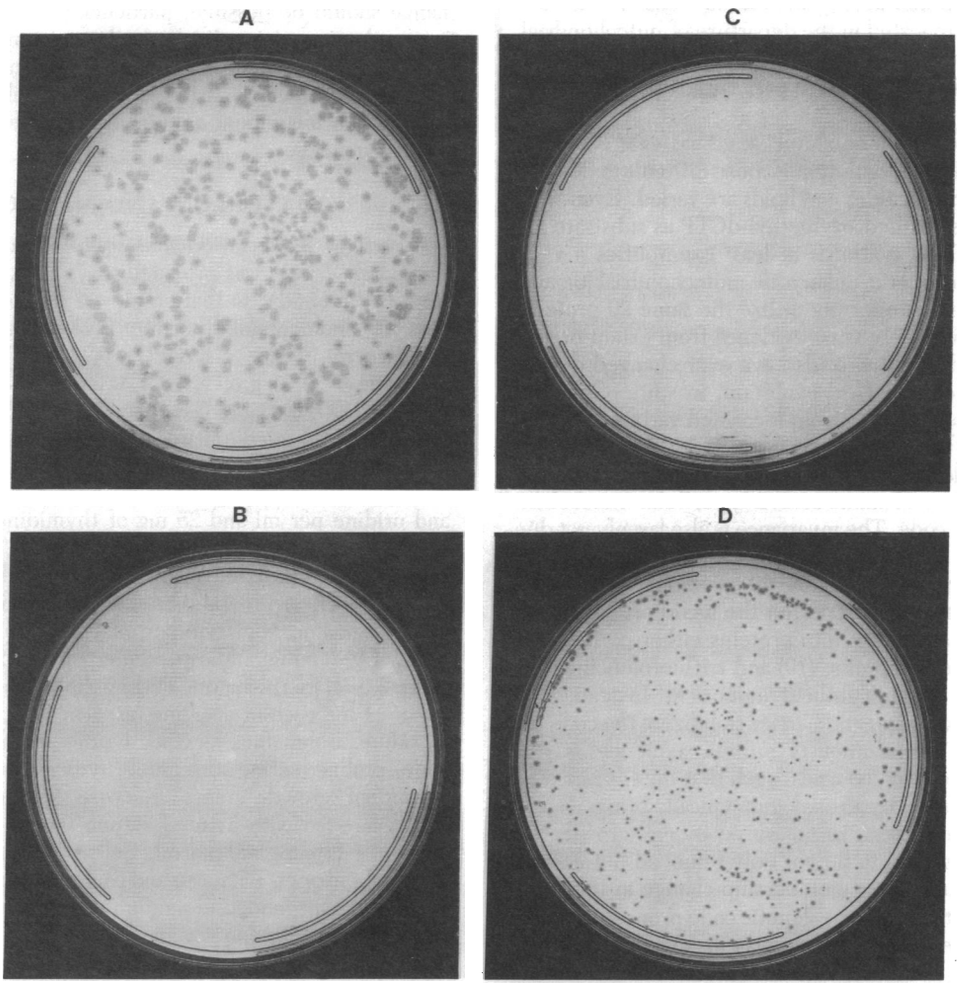

FIG. 1. Growth of strains on agar containing 5 μg of Trp or 4-FTrp per ml. (*A*) QB928 on Trp; (*B*) QB928 on 4-FTrp; (*C*) HR15 on Trp; and (*D*) HR15 on 4-FTrp. *A* was grown for 1 day and *B–D* were grown for 2 days. The same numbers of cells were plated in *A* and *B* and in *C* and *D*.

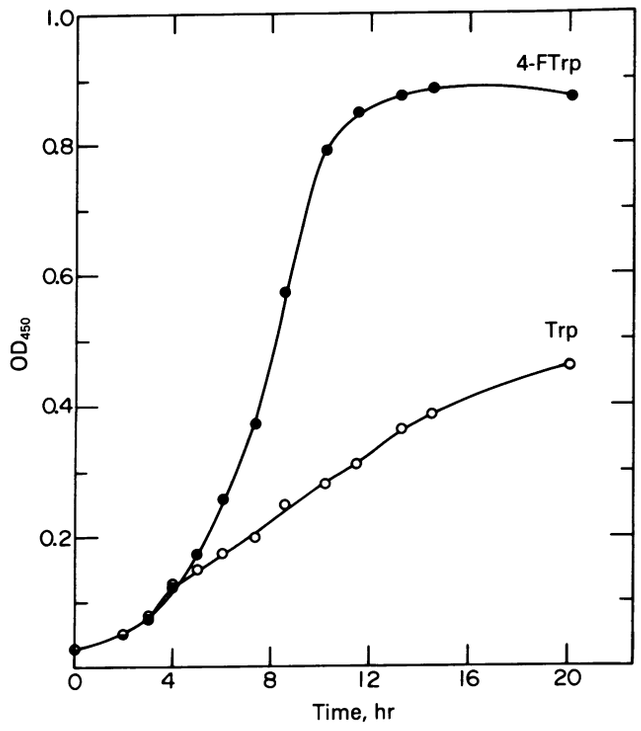

FIG. 2. Growth of HR15 in suspension. Cells in early logarithmic phase in 4-FTrp were harvested by centrifugation and washed five times with prewarmed medium G. They were resuspended in medium G with 5 μg of Trp per ml or medium G with 5 μg of 4-FTrp per ml and were grown on a rotary shaker at 400 rpm.

acteristic on 20 μg of 4-FTrp per ml compared to QB928 or LC8. Strain L33 gave well-formed colonies also at 5 μg of 4-FTrp per ml.

Isolation of Strain HR15. LC33 cells grew more slowly on Trp than QB928 or LC8. This, together with its greater ability to grow on 4-FTrp, led to a reduced divergence in Trp- and 4-FTrp-supported growths compared to QB928 or LC8. Nonetheless, it grew better on Trp than on 4-FTrp. Therefore, the next stage of mutant isolation was directed toward a search for colonies with a high reversed growth preference between Trp and 4-FTrp. For this purpose L33 cells were mutagenized, grown up, and plated on a selective agar containing 0.5 μg of 4-FTrp and 20 μg of Trp per ml, so that cells that were slow to grow on Trp might be expected to yield smaller colonies than other cells. From such small colonies HR7 was obtained, which grew better on 4-FTrp than on Trp. To further reduce Trp-supported growth, HR7 was mutagenized, grown up, and subjected to penicillin treatment. Afterwards the cells were again plated on the same selective agar. One of the small colonies tested was HR15, which showed poor growth on 20 μg of Trp per ml (with a Trp/4-FTrp growth ratio of 0.12 at that concentration) and even poorer growth on 5 μg of Trp per ml.

The Trp/4-FTrp growth ratios for the various strains at 5 μg of Trp and 4-FTrp per ml recorded a sharp decline over the series of mutations from QB928 to HR15 (Table 1). Because L-Trp but DL-4-FTrp were employed, the growth ratio would tend to favor Trp to some extent. The agar plates in Fig. 1 further illustrate the conversion of the unambiguous Trp preference of QB928 to the unambiguous 4-FTrp preference of HR15.

Growth of HR15 in Suspension. HR15 in liquid medium G grew logarithmically in 4-FTrp until stationary phase but became linear after two generations in Trp (Fig. 2). The cessation of logarithmic growth of HR15 in Trp resembled the behavior

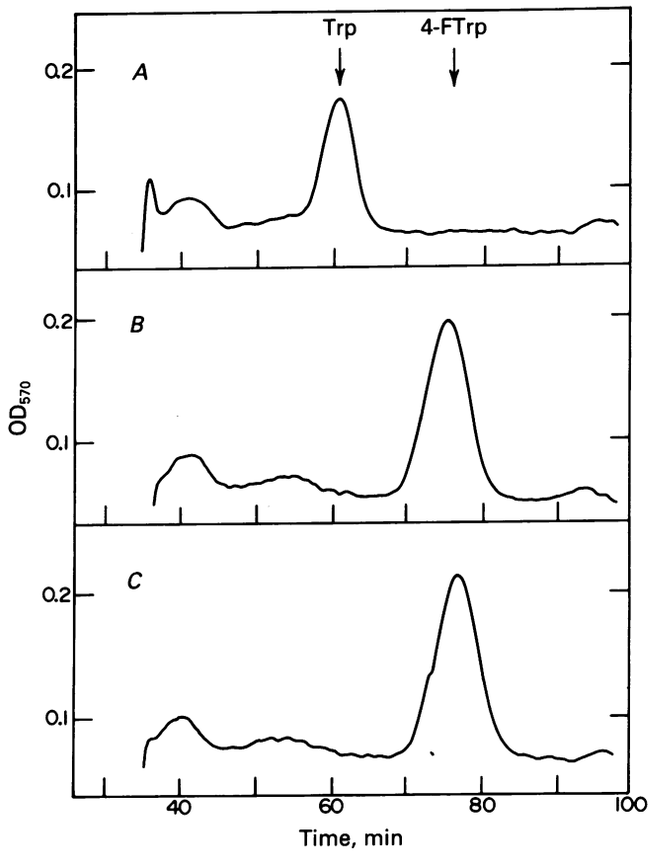

FIG. 3. Amino acid analysis of proteins of LC33 cells grown in 5 μg of Trp per ml (*A*), LC33 cells grown in 5 μg of 4-FTrp per ml (*B*), and HR15 cells grown in 5 μg of 4-FTrp per ml (*C*).

of *Escherichia coli* in 4-FTrp (22, 23). These observations, typical of many amino acid analogues, suggest that Trp was freely incorporated into HR15, just as 4-FTrp was freely incorporated into *E. coli*. In both cases the incorporation of an incompetent amino acid led after a couple of generations to the accumulation of inadequate proteins, dilution of adequate proteins, and the prevention of logarithmic growth.

Amino acid analysis of cell proteins showed that 4-FTrp was incorporated into proteins intact with little, or limited, prior metabolism to Trp (Fig. 3).

DISCUSSION

To mutate the fitness of an encoded amino acid, Trp was chosen as target because of its fairly rare occurrence in cell proteins and the relative tolerance of bacterial cells toward replacement of Trp by its fluorinated analogue 4-FTrp or 6-fluorotryptophan. In *E. coli*, up to 75% of Trp could be replaced by 4-FTrp, although growth eventually stopped after a couple of generations in the absence of Trp (22, 23). From *B. subtilis* QB928, it has been possible to isolate through LC8 the LC33 strain, which utilizes either Trp or 4-FTrp for indefinite growth, and eventually the HR15 strain, which grows well on 4-FTrp but at best marginally on Trp.

It is a tribute to the remarkable stability of the 20-member amino acid code that the question of appropriate criteria for membership hitherto has never arisen. Three useful criteria could help to determine membership in the face of two or more competing amino acids: (*i*) which competitor better supports growth, (*ii*) which competitor is incorporated into proteins at a higher rate, and (*iii*) which competitor is more available either from

the environment or through biosynthesis. Because it does not serve the organism to utilize faster an amino acid that leads to poorer growth, criterion *ii* is expected to be a less important determinant than either *i* or *iii* in guiding membership selection for the amino acid code. In contrast, both *i* and *iii* likely contributed to the ascendance of the universal code. For the Trp-auxotrophic QB928, *iii* is inapplicable, but mutation of Trp membership to 4-FTrp membership might be sought on the basis of the Trp/4-FTrp growth ratio. Under the experimental conditions of Table 1, [^{32}P]phosphate incorporation could be employed readily for the estimation of both extreme and moderate ratios. The Trp/4-FTrp ratio of 730 for QB928 clearly establishes Trp as a competent member amino acid and 4-FTrp merely an inferior analogue. On the same basis, the ratio of 0.036 for HR15 points to a reversal, with 4-FTrp becoming the competent member and Trp the inferior analogue. This change of code membership leads to some immediate conclusions regarding the evolution of the code.

The coevolution theory, though supported by a wide range of evidence (14–18), is opposed by an apparent immutability of code membership throughout the living world. However, the present study rules out the possibility of any inherent immutability. It confirms the coevolutionary postulate that membership in the genetic code, no less than any other biological construct, is both open to, and the result of, evolutionary selection. The enduring stability of the universal code is simply the consequence of its superb performance (15).

A surprising aspect of the present findings is how far the Trp/4-FTrp growth ratio could be decreased through no more than a few mutations. The unmutagenized changes from QB928 to LC8 to LC33 might only be single mutations. The changes from LC33 to HR7 to HR15, because nitrosoguanidine mutagenesis was employed, might incur single or multiple mutations. Nonetheless, altogether just a few serial mutations sufficed to reduce the Trp/4-FTrp ratio from 730 to 0.036—i.e., by a factor of 2×10^4. Evidently, though there are thousands of Trp residues in *B. subtilis* proteins, the functioning of a vast majority of them is equally fulfilled by Trp and 4-FTrp. Therefore, the growth ratio depends on the few nonneutral Trp residues whose tasks are performed unequally well by Trp and 4-FTrp. The transition from QB928 to LC33 likely arose through mutations removing the original bias of some nonneutral Trp residues against performance by 4-FTrp relative to Trp and the transition from LC33 to HR15 through mutations conferring on some Trp residues a newfound bias against performance by Trp relative to 4-FTrp.

It has been argued that the genetic code, once established, will never change (24). The barrier against change turns out to be formidable, but not rigidly insurmountable. The facile takeover of Trp membership by 4-FTrp must have taken place across one of the weakest links of this barrier. Still, the present study has required no selection methods that are unique to the Trp–4-FTrp system. The use of an enriched nutrient medium, to eliminate as much as possible the effect of any poor performance of 4-FTrp in the proteins of nutrient biosynthetic pathways, is a generally applicable approach. Thus, other membership mutations are attemptable—e.g., displacing methionine with selenomethionine, tyrosine with fluorotyrosine, etc. The probability of success will depend in each case on the number of nonneutral protein residues biased against change and the strength of the bias. HR15 may well be the first free-living organism in the past couple of billion years to have learned to thrive on a genetic code that departs from the universal code, especially if the deviant mitchondrial codes represent only simplifications of the universal code in adaptation to a miniature organelle genome, rather than alternative codes serving a full-fledged genome (3, 25). However, it may not be the last.

The performance of proteins or any other class of macromolecules is ultimately restricted by the choice of building blocks. Thus, the genetic code defines the nature of life. With the advent of genetic engineering, protein sequences composed of its 20 standard amino acids can be permutated at will. These permutations provide insight into biological evolution within the confines of a constant amino acid vocabulary but throw no light upon the origin and evolution of the vocabulary itself. Only by making changes in the vocabulary and examining the factors that facilitate or impede such changes, can we hope to understand what shaped the genetic code in the past and how far the genetic code may be redesigned in the future.

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