Synthetic Biology

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Chemical Evolution of a Bacterial Proteome**

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Abstract: *We have changed the amino acid set of the genetic code of Escherichia coli by evolving cultures capable of growing on the synthetic noncanonical amino acid* L*-β-(thieno[3,2-b]pyrrolyl)alanine ([3,2]Tpa) as a sole surrogate for the canonical amino acid* L*-tryptophan (Trp). A long-term cultivation experiment in defined synthetic media resulted in the evolution of cells capable of surviving Trp→[3,2]Tpa substitutions in their proteomes in response to the 20899 TGG codons of the E. coli W3110 genome. These evolved bacteria with new-to-nature amino acid composition showed robust growth in the complete absence of Trp. Our experimental results illustrate an approach for the evolution of synthetic cells with alternative biochemical building blocks.*

Translational ambiguity as an underlying mechanism behind genetic code flexibility is a "sweet spot" for the development and evolution of alternative genetic codes.[1–3] Based on these findings we ventured on the next step towards the experimental diversification of the genetic code: the complete substitution of a canonical amino acid (cAA) by a noncanonical one (ncAA). In contrast to approaches based on stop codon suppression,[4–6] we set out to incorporate ncAAs in a proteome-wide manner. Although previous work in this direction has been conducted,[7–9] the absence of sophisticated analytics and proteomics tools hindered conclusive evidence of full proteome-wide replacement with a noncanonical analogue.

Trp is believed to be evolution's latest addition to the genetic code[10] and demands the highest metabolic synthesis cost of all proteinogenic amino acids. Therefore, it has relatively low abundance in proteins (ca. 1%, that is, about 20000 residues in the whole *E. coli* proteome[11]) and is encoded by a single codon (UGG). It possesses special biophysical properties which allow for its participation in numerous interactions (π→π stacking, hydrogen bonding, cation-π interactions). Therefore, Trp plays a major role in protein stability and folding, and participates in mediation processes such as receptor–ligand interactions and enzyme–substrate binding. Thus, substitution of Trp with other cAAs can often result in misfolded proteins and inactive enzymes, ultimately causing cell death. However, Trp's diverse and rich indole chemistry offers numerous possibilities for potential analogues which might take over Trp's function after cellular and genomic bacterial adaptation. In other words, Trp substitution in proteins with related aromatic systems seems to be plausible without causing totally detrimental effects on the structural and functional integrity of the cell. In the 1950s, the possibility for incorporation of 7-azatryptophan and 2-azatryptophan into proteins was reported.[12,13] However, until recently Trp residues have been mainly replaced in single recombinant proteins by various noncanonical aza-, fluoro-, amino-, and hydroxytryptophan analogues.[14]

On the proteomic scale, Trp-auxotrophic *Bacillus subtilis* strains selected for growth on 4-fluorotryptophan (4FTrp) as a sole substitute for Trp have been reported.[8] However, they were grown in "rich" synthetic medium supplemented with vitamins, nucleobases, and amino acids. These additives can metabolically compensate for potentially deleterious effects of 4FTrp on protein function and lessen the selection pressure for a completely functional modified proteome. Furthermore, these strains were generated by the use of a mutagenic agent hampering conclusive genotype analyses. A different strategy for adapting *E. coli* to 4FTrp consisted of long-term continuous batch culturing by serial dilution[15] in pure mineral medium.[9] During continuous cultivation, the initial Trp content of the medium was decreased stepwise while the

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concentration of 4FTrp was constantly raised. At the end of the long-term cultivation experiment, the resulting strain was able to grow, but only very poorly (end $OD_{600nm} \approx 0.1$) on 4FTrp. However, the impurity of the commercial 4FTrp preparation (i.e. residual traces of Trp) was responsible for a significant residual presence of Trp (≈ 90 nM) in the final culture medium. In the present study, we set ourselves the goal to select an *E. coli* strain capable of growing with a Trp analogue in pure mineral medium without any traces of canonical Trp.

For this purpose, we chose to use [3,2]Tpa as the Trp analogue (Figure 1) in our evolution experiment since its

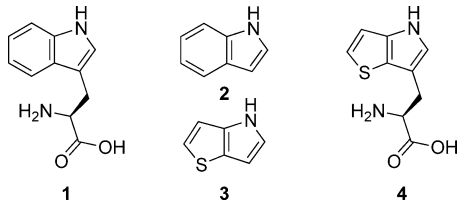

Figure 1. Structures of tryptophan (Trp, **1**), indole (Ind, **2**), β-thieno-[3,2-*b*]pyrrole ([3,2]Tp, **3**), and L-β-(thieno[3,2-*b*]pyrrolyl)alanine ([3,2]Tpa, **4**).

synthetic route excludes any indole or Trp intermediate (see the Supporting Information (SI)). [3,2]Tpa is known to be translationally active and incorporation into single target proteins has already been demonstrated.[16] In this earlier study we had already observed that the replacement of the benzene ring of Trp with thiophene was not only well tolerated by the auxotrophic cells, but [3,2]Tpa was also used to some extent as a substrate for cellular growth.[16]

In the present study we unambiguously confirmed by in vitro and in vivo activation and aminoacylation studies that [3,2]Tpa is a good substrate for *E. coli* tryptophanyl-tRNA synthetase (Section 3 in the SI). Suitable auxotrophic strains are a prerequisite to apply selection pressure for amino acid analogue usage by the cell. We have chosen the *E. coli* K12 W3110 derivative CGSC# 7679.[17] In this strain, the whole Trp biosynthesis pathway is removed (Δ*trpLEDCBA*). In Trp-auxotrophic *E. coli* strains, Trp and its analogues enter the cell via transporter-mediated uptake.[18] This mechanism might be a ready-made target for the cell to shut down analogue uptake and avoid disadvantageous consequences of incorporation into proteins. Therefore, our experimental setup was based on indole (Ind) and β-thieno[3,2-*b*]pyrrole ([3,2]Tp) (Figure 1), which enter bacterial cells by passive diffusion through the membrane.[19] To convert these precursors intracellularly into Trp and [3,2]Tpa, respectively, we equipped CGSC# 7679 with the plasmid pSTB7,[20] which harbors the *Salmonella typhimurium* Trp synthase (TrpBA), an enzyme known to efficiently convert [3,2]Tp into [3,2]Tpa. Our initial strain configuration *E. coli* K12 W3110 (Δ*trpLEDCBA*) ⟨pSTB7⟩ was designated MT0.

We used New Minimal Medium (NMM) throughout the evolution experiment.[21] In the beginning, the medium contained a set of all canonical amino acids except Trp (NMM19) to remove the pathways of amino acid biosynthesis from the initial evolutionary landscape encountered by the cells. Additionally, supplying amino acids leads to higher culture densities facilitating the reduction of indole concentration, and to shorter generation times that are doubtlessly advantageous for initial adaptation. The amino acids were gradually removed at later stages of the experiment to end up with a complete mineral medium and glucose as the sole carbon source (NMM0).

MT0 cells did not grow in the presence of [3,2]Tp (25 μM) without added indole. However, additional supply of only 1 μM of indole was sufficient to obtain a final OD_{600nm} of 0.8–0.9. Starting from this configuration, we conducted a long-term evolution experiment by serially re-inoculating our *E. coli* culture in shaking flasks. The cells were gradually forced to lose their Trp dependence by continuously reducing the amount of indole from the initial 1 μM to zero while maintaining a concentration of 25 μM of [3,2]Tp in the medium (Figure 2).

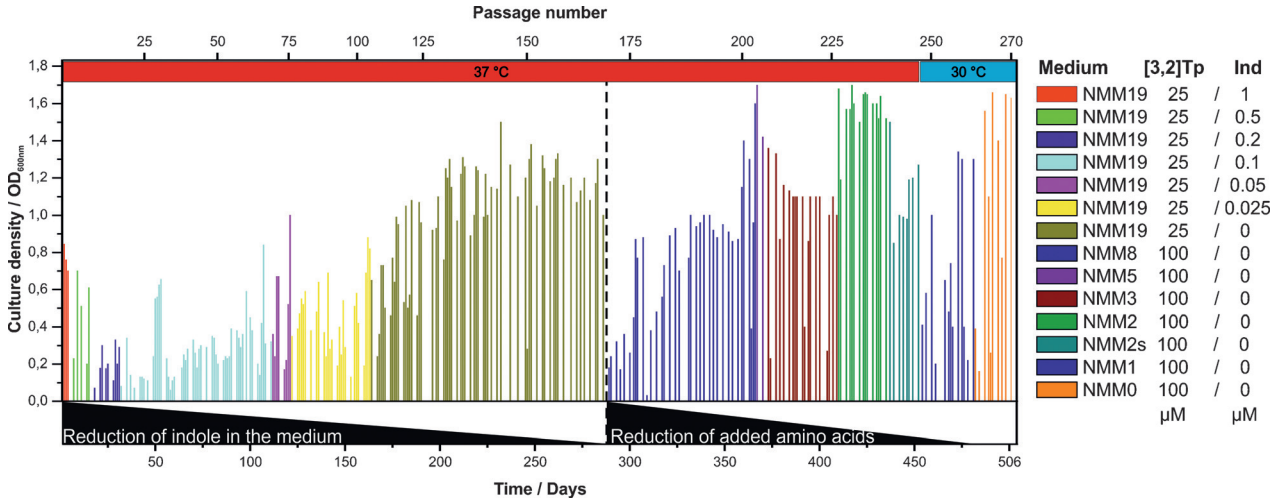

Figure 2. Adaptation of *E. coli* strain CGSC# 7679 ⟨pSTB7⟩ to [3,2]Tpa. The upper scale indicates the passage count while the lower scale shows the time in days. In each passage, OD_{600nm} was measured. The growth temperature was decreased from 37 °C to 30 °C while going from NMM2 to NMM1. The different medium compositions are indicated on the right side and are described in detail in Section 4 in the SI.

Interestingly, already after 106 serial passages (164 days of cultivation) the population was able to grow in NMM19 without any further indole supplementation. After around 150 passages, maximum optical culture densities stopped fluctuating and stabilized around 1.3 OD_{600nm}. With passage 170 (289 cultivation days) we started a stepwise withdrawal of amino acids. To identify amino acids not essential for culture growth on [3,2]Tp, we grouped the 19 canonical amino acids into their metabolic blocks and removed them in a combinatorial manner (Figure 2). Subsequently, within these blocks, some individual amino acids were removed from the medium one by one (Section 5 in the SI). A total of six steps of amino acid removal were required. It has to be pointed out that we were only able to remove the two last amino acids, methionine and histidine, from the medium when we decreased the culture temperature from standard 37°C to 30°C. We hypothesize that [3,2]Tpa incorporation led to misfolding of enzymes important in these amino acid biosynthesis pathways.

Finally, after 264 passages we obtained an *E. coli* population capable of growing in mineral glucose medium (NMM0) supplemented with [3,2]Tp. The full adaptation to growth on [3,2]Tp took place in the time frame of 506 days. At that stage, the culture grew to 1.6 OD_{600nm} in around two days (Figure 2).

The purity and genetic identity of the evolution culture was continuously controlled on agarose plates and by PCR amplification of key genomic regions within the deleted Trp operon and plasmid pSTB7 (Sections 6 and 7 in the SI). The culture was free of contamination and maintained auxotrophy for Trp/[3,2]Tpa throughout the whole evolution experiment.

At different time points of the experiment, we isolated robustly growing clones from the cultures to document the different stages of evolution in our continuous culture (MT strains). The endpoint isolate termed MT20 is capable of growth at 30°C on NMM0 containing only [3,2]Tp while keeping its preference for indole as well. Comparing the growth kinetics of MT0 and MT1 (an isolate originating from the middle stage of the experiment), the difference in generation times in LB at 37°C increases slightly from 24.4 to 28.8 min. It should also be mentioned that the overnight OD_{600nm} decreased from 3.5 in MT0 to 2.2 in MT1. These results indicate that the MT1 strain partially accumulated mutations lowering its relative fitness in LB full medium in comparison to the original MT0 strain.

We also performed initial cell morphological investigations and have found that cells which evolved to grow in the presence of [3,2]Tp present a different phenotype, as shown in Figure 3. In general, evolved MT1 cells exhibit increased length (ca. 1 μm) and reduced width (ca. 0.1 μm). Curiously, MT1 cells grown in NMM19 medium were thinner when grown in presence of indole. This may be a consequence of amino acid starvation processes triggered by the absence of the canonical Trp.[22] We are currently performing comprehensive microscopic and genome analyses in order to shed light on possible physiological mechanisms underlying these changes in fitness.

To quantify [3,2]Tpa incorporation at Trp positions in the proteome and to exclude the possibility of trace Trp presence

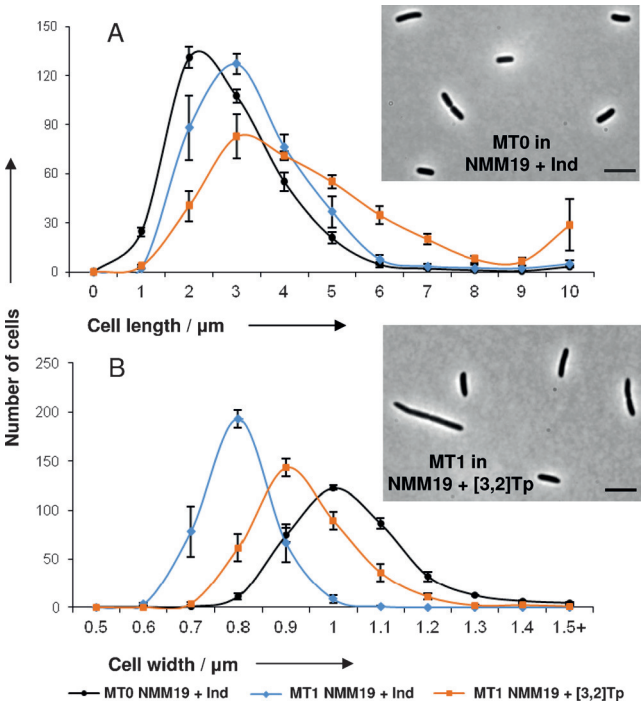

Figure 3. Morphology and average distribution of cell length (A) and width (B) as measured by microscopy of MT0 and MT1. Strains were grown in NMM19 with either 100 μM of indole (black dots: MT0; blue diamonds: MT1) or of [3,2]Tp (orange squares: MT1). Error bars represent the standard errors of the means of four experiments (with 350 cells studied by experiment). Inserts: Examples of brightfield microscopy pictures of MT0 (0 μM [3,2]Tp/100 μM indole) and MT1 (0 μM Indole/100 μM [3,2]Tp) cells grown in NMM19 and prepared on a 1% agarose-coated slide to be visualized by microscopy. The calibration bar indicates 5 μm. Details can be found in Sections 9 and 10 in the SI.

or genetic strain reversion, we carried out highly sensitive analytics using gas chromatography and MS/MS measurements. We chose re-grown MT isolates in their respective culture conditions as samples for these analyses (Table 1 and Sections 5 and 9 in the SI).

MS/MS analyses were conducted using individually grown cultures in two different laboratories using distinct MS/MS techniques (one example is shown in Figure S5). Both analyses successfully provided direct evidence for a full Trp→[3,2]Tpa substitution throughout the whole proteome. We analyzed in detail the 20 most abundant peptides containing a single Trp position and emerging from different proteins (Section 8 in the SI). Computer-aided MS/MS data analyses showed that cell cultures grown exclusively on [3,2]Tp incorporated the ncAA into their proteome with no Trp identification in peptides. In addition, heterologous expression of eGFP in [3,2]Tp-containing medium resulted in completely labeled protein as verified by ESI-MS analysis (Section 7 in the SI).

The application of very sensitive GC/MS analyses enabled us to unambiguously verify the complete liberation of the proteome from Trp (Table 1). Interestingly, in cultures of isolates MT1 and MT12, traces of Trp were still detected in the proteome even though indole was no longer added to the

Table 1: Proteome-wide [3,2]Tpa incorporation followed by mass spectrometric and amino acid analyses.

Strain[b]	MT0	MT0	MT1	MT20
MS/MS:[c]				
Trp [%]	99.5 ± 1.2	99.7 ± 0.5	0.6 ± 0.8	0.5 ± 0.8
[3,2]Tpa [%]	0.5 ± 1.2	0.6 ± 0.5	99.4 ± 0.8	99.5 ± 0.8
Amino acid analysis:[d]				
Trp [%]	100 ± 3.8	98.7 ± 3.2	1.6 ± 0.1	–[e]
Medium:				
Aa conc.[f]	19	19	19	0
Ind [μM]	100	100	0	0
[3,2]Tp [μM]	0	25	25	100

[a] The MT strains were isolated at different times throughout the long-term evolution experiment (Figure 2) and represent increasing stages of adaptation. An extended version of this table with additional intermediates can be found in the SI (Table S5). [b] Strain details are provided in Sections 4 and 6 in the SI. [c] Measured peptide occurrence of [3,2]Tpa and Trp measured by MS/MS. 20 of the most abundant tryptophan-containing peptides were chosen for quantification. [d] GC/MS analysis of hydrolyzed proteome samples derivatized by esterification and amidiation. [e] No peak observed in the chromatogram. [f] Amino acid content of the medium. A detailed overview of the medium composition can be found in Section 4 in the SI.

culture media. Trace amounts of Trp may be explained by a contamination of medium preparations. This should not be surprising as the majority of commercially available amino acids are preparations from biological fermentation processes.

With the full removal of all amino acids from the medium, the residual Trp traces completely disappeared (MT16–MT20). Therefore, in the frame of the detection limits of our methodology and the available instrumentation, these GC/MS analyses demonstrate the complete proteome-wide absence of Trp traces in the evolved cells. It should be kept in mind that we could not provide direct evidence for the presence of [3,2]Tpa by GC/MS analysis because it is readily degraded in the hydrolysis step of the amino acid analysis. This is not surprising as thieno surrogates of Trp are less stable (e.g., the aromatic delocalization energy of benzene is 36 kcal mol^{-1} whereas thiophene and thiazole have only 29 kcal mol^{-1} and 25 kcal mol^{-1}, respectively)[23].

We have generated an organism whose proteomic composition changed from initial Trp to [3,2]Tpa during a long-term evolution experiment without significant negative impact on its cellular survival. This uncovered a high evolutionary plasticity that has allowed the substitution of all the tryptophan in the proteome of *E. coli*. We are currently analyzing genomes of evolved strains and anticipate (based on previous work with fluorinated Trp analogues[8,9]) that only a relatively small number of proteins should be detrimentally affected by [3,2]Tpa incorporation.[24]

Genetic encoding in living systems is based on highly standardized chemistry composed of the same number of "letters" or nucleotides as informational polymers (DNA, RNA) and the twenty α-amino acids as basic building blocks for proteins. The universality of the genetic code enables the horizontal transfer of genes across biological taxa which affords a high degree of standardization and interconnectivity. Thus, all deep chemistry changes within living systems tend to be generally lethal.[25–27]

In this context, one of the great challenges for 21st century bioscience is the development of a strategy for expanding the standard basic chemical repertoire of living cells to achieve biocontainment by man-made or naturally evolved changes in the genetic code.[28–30] Recently, the non/canonical chemical barrier was crossed by evolving an *E. coli* strain with a genome in which thymine was replaced by 5-chlorouracil.[31] This is a first important example in the construction of biological systems composed of xenonucleic acids.

Based on the notion that between 30 and 40 sense codons are adequate to encode the genetic information of an organism,[32] a large number of sense codons (> 20) may be available for recoding with ncAAs. Current "genetic code expansion" approaches,[33] programmed to reassign UAG or UGA stop codons, are geared to produce modified proteins containing one or multiple ncAAs with fluorescent or chemically reactive groups for a host of in vivo or in vitro applications. However, proteome-wide replacements of amino acids are not practical by this route. Our long-term *E. coli* evolution experiment resulted in an organism in which all 20899 UGG codons were "recoded" by substituting the supply of the original Trp-tRNATrp with a ncAA-tRNA (i.e. [3,2]Tpa-tRNATrp) formed by the same aminoacyl-tRNA synthetase. This example of a proteome-wide amino acid replacement represents a significant "genetic code expansion" and a real step towards a synthetic organism.

Taken together, approaches to alter the meaning of the genetic information stored in DNA as an informational polymer by changing the chemistry of the polymer (i.e., xenonucleic acids) or by changes in the genetic code have already yielded successful examples.[34] In the future this should enable the partial or full redirection of the biological information flow to generate "new" version(s) of the genetic code derived from the "old"[25] biological world.

Keywords: continuous evolution · *Escherichia coli* · genetic code translation · synthetic biology · tryptophan analogues

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