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A Bacterial Strain with a Unique Quadruplet Codon Specifying Non-native Amino Acids

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The addition of noncanonical amino acids to the genetic code requires unique codons not assigned to the 20 canonical amino acids. Among the 64 triplet codons, only the three nonsense "stop" codons have been used to encode non-native amino acids. Use of quadruplet "frame-shift" suppressor codons provides an abundant alternative but suffers from low suppression efficiency as a result of competing recognition of their first three bases by endogenous host tRNAs or release factors. Deletion of release factor 1 in a genomically recoded strain of *E. coli* (*E. coli* C321), in which all endogenous amber stop codons (UAG) are replaced with UAA, abolished UAG mediated translation termination. Here we show that a *Methanocaldococcus jannaschii*-derived frame-shift suppressor tRNA/aminoacyl-tRNA synthetase pair enhanced UAGN suppression efficiency in this recoded bacterial strain. These results demonstrate that efficient quadruplet codons for encoding non-native amino acids can be generated by eliminating competing triplet codon recognition at the ribosome.

With rare exceptions, proteins in all domains of life are biosynthesized from the same 20 canonical amino acids, which are encoded by 61 triplet codons. The expansion of this standard genetic code to allow the site-specific introduction of noncanonical amino acids (ncAAs) into proteins in both prokaryotic and eukaryotic organisms has provided a powerful new tool to both probe and manipulate protein structure and function.[1] The ncAA of interest is encoded by a nonsense or frame-shift codon and is co-translationally incorporated by using a cognate tRNA/aminoacyl-tRNA synthetase (aaRS) pair.[1] This technology currently allows the incorporation of up to two distinct ncAAs into a single polypeptide chain.[2–8] The ability to simultaneously encode many distinct ncAAs for in vivo protein translation would further expand the scope of this technology and might ultimately permit the ribosomal biosynthesis of entirely unnatural biopolymers with novel properties.[6] Each distinct ncAA to be used in such a system would require a dedicated "blank" codon and a corresponding orthogonal tRNA/aaRS pair (i.e., a tRNA/aaRS pair that does not cross react with either exogenous or host tRNAs and aaRSs).[1] To this end, several mutually orthogonal tRNA/aaRS pairs have been developed for use in *E. coli*[1,2,9,10] that efficiently suppress different nonsense and quadruplet codons.[1,2,9,11] In addition, the incorporation of two distinct ncAAs into proteins in *E. coli* has been carried out by using two mutually orthogonal tRNA/aaRS pairs suppressing either two different nonsense codons[3,4,7,8] or a combination of a nonsense and a frame-shift codon.[2,5] However, only two nonsense codons can be used to encode ncAAs, and although frame-shift codons offer an abundant alternative,[12–14] their suppression efficiency tends to be significantly lower than that of their triplet counterparts. This is likely due to a combination of less efficient translation by the ribosome and competing recognition of their first three bases, leading to undesired triplet suppression or termination (Figure 1). Recently,

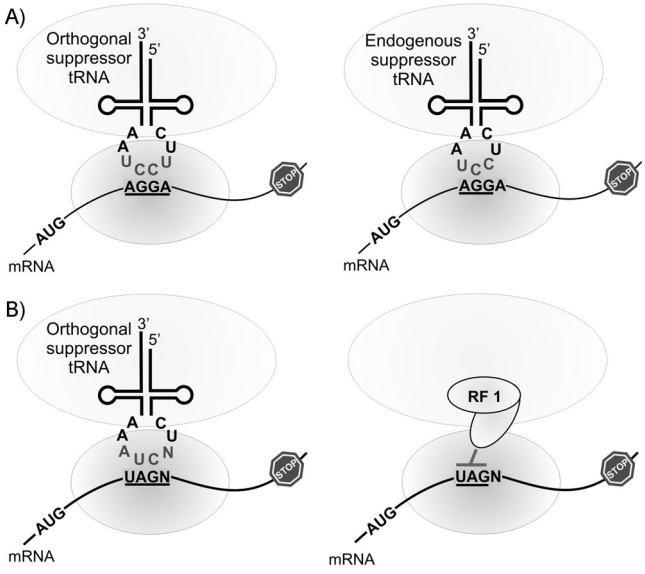

Figure 1. Competing triplet recognition during the translation of quadruplet codons. A) For most quadruplet codons such as AGGA, suppression of its first three bases by an endogenous tRNA (right; in this case an arginyl tRNA suppressing AGG) competes with the desired quadruplet suppression (left). B) For UAGN quadruplet codons, competing recognition of UAG by RF1, leading to translation termination (right), competes with desired quadruplet suppression (left).

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ribosome engineering was used to enhance the suppression efficiency of a quadruplet codon, presumably by improving its ribosomal processing.[5] However, this approach fails to eliminate the competing triplet recognition and associated loss in efficiency.

An analysis of the effects of competing triplet codon recognition on quadruplet codon suppression efficiency has remained a challenge, as it requires the availability of a host where the use of the corresponding triplet codon, as well as the mechanism responsible for its suppression/termination, is absent. Recently, multiplex automated genome engineering (MAGE) and conjugative assembly genome engineering (CAGE) were used to replace all instances of the UAG amber nonsense codon (321 total) with the UAA ochre nonsense codon in the *E. coli* MG1655 genome.[15] In the absence of endogenous UAG codons, release factor 1 (RF1) is not required and can be removed without an associated fitness penalty. In the resulting strain, UAG is no longer recognized as a nonsense codon and has been used to uniquely and efficiently encode unnatural amino acids in the presence of the cognate amber suppressor tRNA/aaRS pair.[15] Here, we investigated how the suppression efficiency of UAGN quadruplet codons is affected by the presence or absence of UAG-mediated translation–termination in this genomically recoded strain of *E. coli* (Figure 1B). Because previous studies have shown that frame-shift codons with the highest suppression efficiencies in *E. coli* begin with a triplet codon that is inefficiently suppressed (e.g., AGGA and CCCU, corresponding to rare arginine and proline codons AGG and CCC, respectively), or with a nonsense codon, we hypothesized that UAGN codons could be very efficiently translated in this strain.[12,13] Indeed, we found that RF1 deletion dramatically enhances the suppression efficiency of the UAGN codons, mediated by a *Methanocaldococcus jannaschii*-derived tyrosyl tRNA/aaRS pair.[1]

To carry out these experiments, one requires a cognate tRNA/aaRS pair that is able to suppress UAGN frame-shift codons in *E. coli*. Because the anticodon is often an identity element in the tRNA-aaRS interaction, the generation of non-native tRNA variants by mutating the anticodon can lead to attenuated aminoacylation activity.[9,16] Thus, efficient frame-shift suppressors are typically generated from tRNA/aaRS pairs that do not exhibit anticodon recognition (such as leucyl, glutamyl, or seryl).[12–14] However, in *E. coli*, the only orthogonal amber suppressor tRNA/aaRS pairs that have successfully evolved to genetically encode a large number of ncAAs are the *M. jannaschii*-derived tyrosyl pair and the archaeal pyrrolysyl pairs.[1] We sought to identify variants of the former pair that can suppress UAGN quadruplet codons, due to its high intrinsic activity[7] and the availability of many mutant aminoacyl-tRNA synthetases with distinct ncAA specificities. For the initial experiments, we used a mutant *M. jannaschii*-derived tyrosyl-tRNA synthetase (MjTyrRS)/$tRNA^{Tyr}$ pair, exhibiting a high degree of polyspecificity for ncAAs but not for the canonical 20 amino acids.[17]

This polyspecific MjTyrRS/$tRNA^{Tyr}$ pair was expressed from the recently reported highly efficient suppressor plasmid pUltra (pUltra-MjTyr), harboring a CloDF13 origin of replication that is compatible with the reporter plasmid.[7] For convenient analysis of suppression efficiency, we chose the green fluorescent protein GFPuv as the reporter, which was expressed following a strong isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T5-*lac* promoter. This expression cassette was incorporated into a pET22-derived vector harboring a pMB1 origin of replication. The permissive Tyr151 site of GFPuv, which has been previously shown to tolerate a variety of different ncAAs without perturbing its folding or fluorescence, was mutated to introduce UAGA, UAGG, UAGU, and UAGC quadruplet codons by site-directed mutagenesis. The CUA anticodon in the MjTyr-derived tRNA was also mutated to generate the corresponding frame-shift suppressor tRNAs that form Watson–Crick base pairs with each of the four UAGN quadruplet codons (Figure 2A). The resulting suppressor tRNAs contain an extended, eight-nucleotide-long anticodon loop. In addition to suppression experiments on tRNAs with perfectly complementary anticodons to the four UAGN quadruplet codons, the following codon/anticodon wobble pairs were also evaluated: GFP(151UAGG)/$tRNA_{UCUA}$ and GFP(151UAGU)/$tRNA_{GCUA}$ (Figure 2A).

In order to assay suppression efficiency, the mutant reporter plasmids were co-transformed with the appropriate pUltra-MjTyr suppressor plasmid into the *E. coli* DH10B cell line. Only successful suppression of the quadruplet codon results in expression of full-length GFP, which can be detected and quantified by using its characteristic fluorescence. Expression of GFP with these strains was performed in 2×YT medium in the presence or absence of 1 mM *p*-acetylphenylalanine (pAcF), an efficient substrate for the polyspecific MjTyrRS. For comparison with amber suppression efficiency, we also generated variants of the same reporter and suppressor plasmids: GFP(151TAG) and pUltra-$MjTyr_{CUA}$, co-transformed them into DH10B, and used the resulting strains to perform GFP expression under identical conditions. Among the different UAGN codon/suppressor combinations tested, GFP(151TAGA)/pUltra-$MjTyr_{UCUA}$ demonstrated the highest expression levels (Figure 2B). The same suppressor plasmid was also able to suppress the UAGG codon by wobble pairing, although at a much weaker level (Figure 2B). Other combinations of reporter/suppressor plasmids failed to express detectable levels of full-length GFP above the control (suppression experiments in the absence of ncAA). However, the suppression efficiency of even the UAGA was significantly weaker (< 5%) relative to its UAG suppressing counterpart. The reduced efficiency is likely due to the competing recognition of UAG at the ribosome and the consequent translation termination (Figure 1B). However, it is also possible that the UAGN suppressor tRNA/aaRS pairs used in this experiment are weakly active.

To evaluate the contribution of competing RF1-mediated translation/termination to the efficiency of UAGN suppression, we repeated the above experiments in two otherwise identical strains of *E. coli*—one with and one without a functional RF1 (C321 and C321.ΔA, respectively).[15] All endogenous UAG stop codons in these strains were replaced with the ochre stop codon UAA, which permits the elimination of RF1 without any adverse effect. In the absence of RF1, C321.ΔA is unable to process the UAG codons encoding an unnatural amino acid as

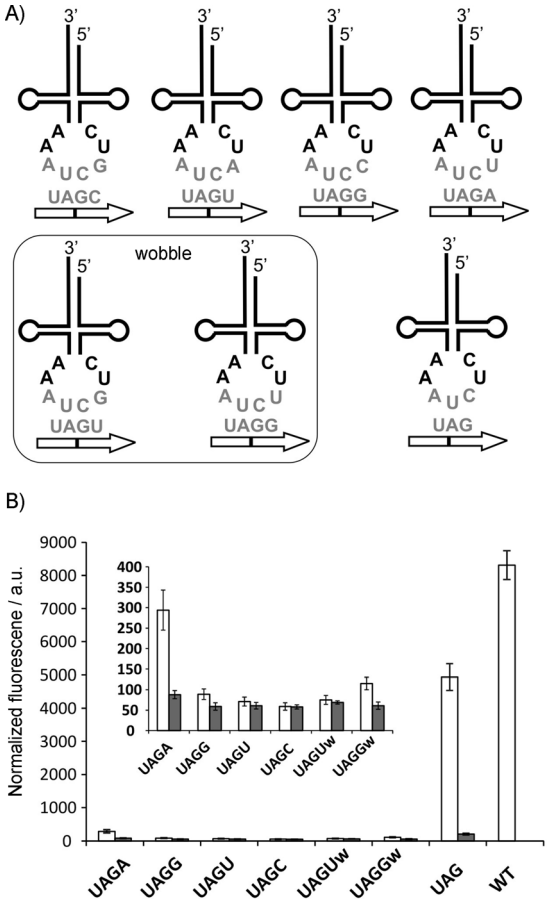

Figure 2. Suppression efficiency of UAGN quadruplet codons by using engineered *M. jannaschii*-derived tyrosyl tRNA/aaRS pairs in DH10B cells. Expression levels of full-length GFP(Tyr151UAGN), as measured by normalized cellular fluorescence, were used to estimate the suppression efficiency of the UAGN quadruplet codon by a cognate UAGN-suppressor MjTyr-tRNA/aaRS. The GFP reporter was expressed from a pET22-derived plasmid; a polyspecific MjTyrRS[17] and the appropriate anticodon variant of $tRNA^{Tyr}$ were expressed from the suppressor plasmid pUltra.[7] Expression of GFP was performed in the presence or absence of 1 mM *p*-acetylphenylalanine (pAcF). A) Different combinations of GFP(Tyr151UAGN)/$tRNA^{Tyr}_{NCUA}$ tested in this experiment. B) Suppression efficiency of various UAGN codons, as well as that of UAG, measured as normalized cellular florescence derived from the expression of full-length GFP(Tyr151TAGN) or GFP(Tyr151TAG) in the absence (■) or presence (□) of 1 mM pAcF. Wobble suppression of UAGU and UAGG are indicated as UAGUw and UAGGw, respectively. Expression level of wild-type GFP (no suppression) is also shown. Indicated data are magnified in the inset.

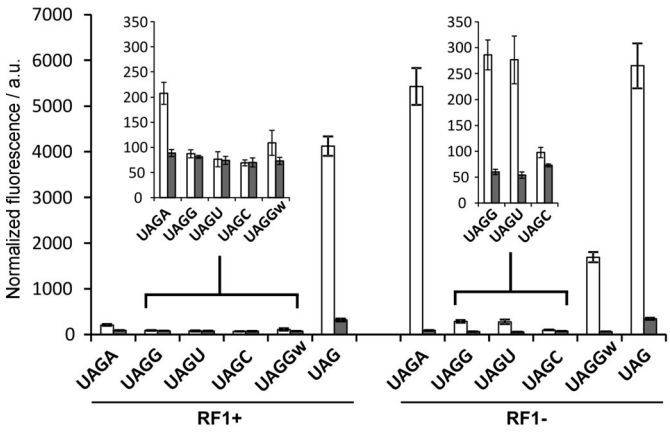

Figure 3. Deletion of RF1 improves UAGN suppression levels. RF1 is deleted from the genomically recoded *E. coli* MG1655 strain C321, lacking any endogenous UAG codons. Suppression efficiencies of the UAGN codons, as well as of UAG, in the C321 strain encoding (RF1+) or lacking (RF1−) RF1 were evaluated as the normalized cellular fluorescence resulting from the expression of full-length GFP(Tyr151TAGN or Tyr151TAG) by using the appropriate pUltra-$MjTyr_{NCUA/CUA}$ suppressor plasmid, in the absence (■) or presence (□) of 1 mM pAcF. Indicated data are magnified in the inset.

a stop codon. GFP(151UAGN) reporter plasmids were co-transformed into these strains, along with the appropriate suppressor plasmids, and the expression level of GFP was monitored as previously described in the presence or absence of 1 mM pAcF. Deletion of RF1 led to an overall increase in suppression efficiency of UAGN codons, resulting in higher GFP(151UAGN) expression levels in the C321.ΔA strain relative to C321 (Figure 3). In particular, expression levels associated with GFP-(151UAGA) and GFP(151UAGG) were very high when suppressed by using pUltra-$MjTyr_{UCUA}$. The expression level of GFP-(151UAGA) in the C321.ΔA strain was similar to that of the GFP amber mutant (151UAG), with a more than 25-fold improvement in suppression efficiency relative to the same in the C321 strain (Figure 3). In agreement with previous reports, RF1 deletion did not significantly improve the suppression efficiency of the UAG triplet codon. This is likely due to the use of a highly efficient expression system[7] in which the amber suppressor tRNA outcompetes RF1.[15]

A comparison of UAGA suppression efficiency among DH10B, wild-type MG1655 (harboring all endogenous TAG codons and a functional RF1), C321 (RF1+), and C321.ΔA (RF1−) *E. coli* strains by using pET22-T5*lac*-GFP(151UAGA)/pUltra-$MjTyr_{UCUA}$ plasmids revealed a high level of protein expression only with C321.ΔA, the RF1− strain (Figure 4A and 4B). By using a C-terminal His_6 tag, full-length GFP was isolated from these cultures grown in 2xYT medium in shaker flasks. The C321.ΔA strain yielded 27 mg L^{-1} of pure mutant protein (approximately 60% of wild-type GFP), demonstrating the high suppression efficiency of the UAGA codon in this strain, whereas the other strains failed to yield demonstrable amounts of protein (Figure 4B). ESI-MS analysis of the purified protein revealed a mass of 27737 Da, consistent with the incorporation of pAcF (Figure 4C). We further tested the UAGA suppression efficiency in the C321.ΔA strain by using superfolder GFP constructs pET22-T5*lac*-sfGFP(151UAGA) and pUltra-$MjTyr_{UCUA}$ in the presence of 1 mM pAcF, which yielded full-length protein at 58 mg L^{-1}. SDS-PAGE and MS analysis of the purified protein was consistent with a homogeneous species incorporating one pAcF (Figure S1 in the Supporting Information).

Interestingly, the observed suppression efficiency of GFP-(151TAGG) when using $MjTyr_{CCUA}$ was substantially lower than that observed with $MjTyr_{UCUA}$ (wobble suppression; Figure 2A), implying a deficiency associated with $tRNA^{Tyr}_{CCUA}$, rather than poor intrinsic suppression efficiency of the UAGG codon (Figure 3). It might be that the anticodon variant of $tRNA^{Tyr}_{CCUA}$ suffers an attenuated charging efficiency relative to $tRNA^{Tyr}_{UCUA}$. MjTyrRS does bind the anticodon loop of $tRNA^{Tyr}$, and muta-

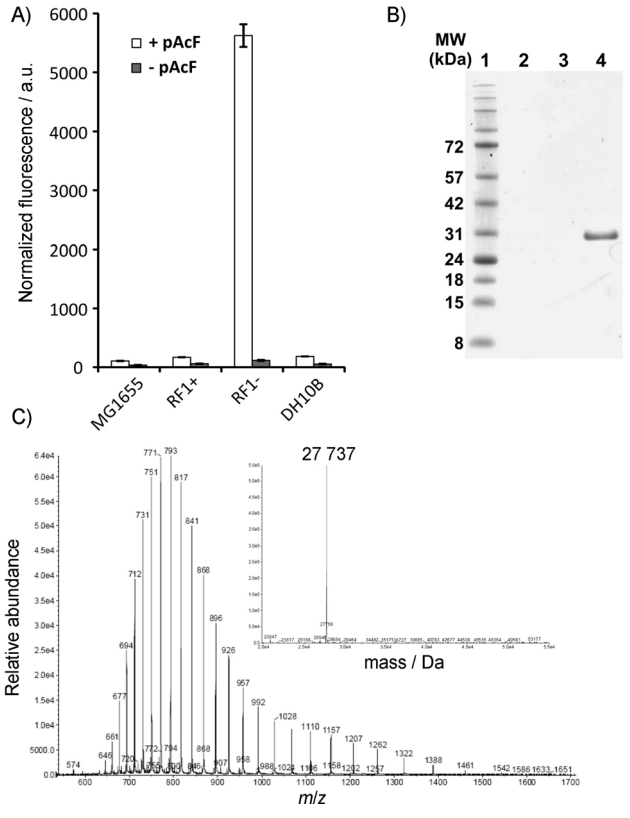

Figure 4. Expression level of full-length GFP(151UAGA) by using pUltra-MjTyrRS(polyspecific)/$tRNA^{Tyr}_{UCUA}$ in various *E. coli* strains, analyzed by A) normalized cellular fluorescence, and B) SDS-PAGE analysis following Ni-NTA purification. RF1+ and RF1− correspond to genomically recoded C321 strains either encoding or lacking RF1, respectively. B) Lane 1, MW marker; lane 2, protein isolated from DH10B; lane 3, protein isolated from RF1+ C321; lane 4, protein isolated from RF1− C321.ΔA. C) ESI-MS analysis of GFP-(151pAcF) isolated from an RF1 strain. Observed mass (27 737 Da) correlates well with the expected mass (27 736 Da).

tions in its anticodon binding site have been shown to enhance amber suppression efficiency of $tRNA^{Tyr}_{CUA}$.[16] It might be possible to improve the performance of the less active MjTyrRS/$tRNA^{Tyr}_{NCUA}$ pairs described here by engineering the anticodon binding site of MjTyrRS in a similar manner. Importantly, experiments described in this report were performed on *E. coli* strains with a wild-type ribosome, demonstrating the ribosome's ability to efficiently translate quadruplet codons. However, it might be possible to further improve the suppression efficiency of UAGN codons by introducing beneficial mutations in the ribosome.[5]

Our results demonstrate that elimination of competing triplet recognition in *E. coli* can lead to significant improvement in the suppression efficiency of quadruplet codons. Recently, it has been shown that 13 rare sense codons can be completely replaced in 42 highly expressed essential genes of *E. coli* by MAGE-mediated introduction of silent mutations.[18] If such experiments can be successfully implemented on a genome-wide scale, followed by deletion of the corresponding cognate tRNAs, it might be possible to create additional triplet codons with no associated "sense"/coding information. The above results suggest that the suppression efficiency of quadruplet codons beginning with such "blank" triplet codons will also be high. In conclusion, the improved performance of the quadruplet codon UAGA demonstrated in this report, comparable to that of UAG—the gold standard for incorporating unnatural amino acids—underscores the utility of genomically redesigned strains of *E. coli* for further expanding the genetic code by using sets of noncoding three- and four-nucleotide codons and their cognate suppressor tRNA/aaRS pairs.

Experimental Section

All chemicals were purchased from commercial sources and used without further purification. PCR reactions were performed with Platinum-pfx polymerase (Life Technologies) following the manufacturer's protocol. DNA oligomers were obtained from Integrated DNA Technologies, and restriction enzymes and T4 DNA ligase were from New England Biolabs. Plasmid DNA was purified by using a ZR plasmid miniprep kit (Zymo). Macherey–Nagel Nucleospin columns were used to purify DNA following digestion or gel electrophoresis. Protein mass spectra were acquired at the Scripps Center for Mass Spectrometry (La Jolla, CA). *E. coli* DH10B was used for routine cloning and DNA propagation. Site-directed mutagenesis was performed by using the Agilent QuikChange-Lightning mutagenesis kit.

Plasmid construction: To construct the GFP expression plasmid pET22b-T5*lac*-GFP, a T5*lac*-GFPuv cassette from the previously reported pLeiG plasmid was amplified and inserted between the NotI and XbaI sites of pET22b (Novagen). GFPuv in this plasmid was replaced by digesting it with NdeI/HindIII, and a superfolder GFP gene was inserted to generate pET22b-T5*lac*-sfGFP. Tyr151 in pET22b-T5*lac*-GFP was mutated to various UAGN codons by using the QuikChange Lightning multisite-directed mutagenesis kit and the appropriate mutagenic oligonucleotide (Table S1). The previously reported suppressor plasmid pUltra, encoding an *M. jannaschii*-derived polyspecific tyrosyl-tRNA synthetase/tRNA, was mutated by using appropriate oligonucleotides (Table S1) to generate variants suppressing different UAGN codons.

Protein expression and purification: The particular *E. coli* strain under investigation, carrying the expression plasmid pET22-T5*lac*-GFP (Tyr151UAGN) and the corresponding pUltra suppressor plasmid, was grown in 2×YT rich medium until the OD_{600} reached 0.6–0.8, at which point the expression of GFP, as well as the synthetase, were coinduced with IPTG (1 mM). At this point, pAcF was added at a final concentration of 1 mM. Protein expression was continued for 16 h at 30 °C. For rapid analysis of GFP expression, cells from an aliquot of culture (100 μL) were resuspended in PBS (200 μL, Mediatech), transferred to 96-well black clear-bottom assay plates, and GFP fluorescence was measured (λ_{ex} = 390 nm, λ_{em} = 509 nm) by using a SpectraMax GeminiEM plate reader (Molecular Devices). Fluorescence readings were normalized by dividing by the corresponding OD_{600} value.

To isolate overexpressed proteins, cells from aliquots of culture (10–40 mL) were harvested by centrifugation and resuspended in BugBuster protein extraction reagent (0.5–2 mL), supplemented with Complete Mini EDTA-free protease inhibitor cocktail (Roche), lysozyme (0.5 mg mL^{-1}), and benzonase nuclease (5 U mL^{-1}, Sigma–Aldrich), and incubated on ice for 15 min. The lysate was clarified by centrifugation at 18 000 *g* for 15 min, and the His_6-tagged protein (C-terminal) was isolated by using Ni-NTA Agarose Superflow resin (Qiagen) according to manufacturer's instructions. The molecular weights of purified proteins were verified by ESI-MS analysis.

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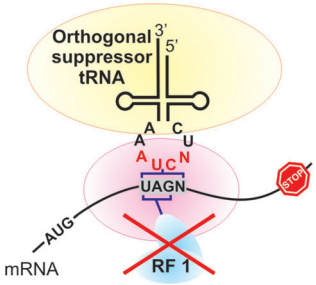
A Bacterial Strain with a Unique Quadruplet Codon Specifying Non-native Amino Acids



Not the usual: Incorporation of unnatural amino acids into proteins in response to quadruplet codons is inefficient, due to competing triplet recognition on the ribosome. Here we demonstrate a dramatic improvement in the suppression efficiency of the UAGA codon, when competing UAG recognition is eliminated by RF1 deletion in a genomically recoded *E. coli* strain devoid of endogenous UAG codons.