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New Methods Enabling Efficient Incorporation of Unnatural Amino Acids in Yeast

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Unnatural amino acids (UAAs) with novel chemical and physical properties have been genetically encoded in living cells by using orthogonal tRNA–codon–synthetase sets.^{1,2} Application of these UAAs for studying biological processes or for industrial and pharmaceutical use would strongly depend on their incorporation efficiencies. Although tens of milligrams of UAA-containing proteins could be produced from 1 L of *Escherichia coli* culture,^{2,3} the yield in yeast was only tens of micrograms.⁴ One challenge is to express orthogonal bacterial tRNAs in yeast because yeast and bacteria differ significantly in tRNA transcription and processing.⁵ We developed a new method to express different orthogonal bacterial tRNAs in yeast with high activity. In addition, mRNA stability of the target gene is a unique, unaddressed issue for UAA incorporation in yeast. The nonsense-mediated mRNA decay (NMD) pathway mediates the rapid degradation of mRNAs that contain premature stop codons in yeast,⁶ whereas no such pathway exists in *E. coli*. When stop codons are used to encode UAAs, NMD could result in a shorter lifetime for the target mRNA and thus a lower protein yield in yeast. We generated an NMD-deficient yeast strain and show here that this strain indeed increases the UAA incorporation efficiency in comparison to the wild-type (wt) yeast. These new strategies enabled UAAs to be incorporated into proteins in yeast in high yields of tens of milligrams per liter.

E. coli tRNAs are transcribed by the sole RNA polymerase (Pol) through promoters upstream of the tRNA gene. However, the transcription of yeast tRNAs by Pol III depends principally on promoter elements within the tRNA known as the A- and B-box (Figure 1a).⁵ The A- and B-box identity elements are conserved among eukaryotic tRNAs but are lacking in many *E. coli* tRNAs. Creating the consensus A- and B-box sequences in *E. coli* tRNAs through mutation could cripple the tRNA.⁷ In addition, all *E. coli* tRNA genes encode full tRNA sequences, whereas yeast tRNAs have the 3'-CCA trinucleotide enzymatically added after transcription.⁵ Therefore, transplanting *E. coli* tRNA gene into the tRNA gene cassette in yeast does not generate functional tRNA. We reasoned that *E. coli* tRNAs could be efficiently expressed in yeast using the following strategy: A promoter containing the consensus A- and B-box sequences is placed upstream of the *E. coli* tRNA to drive transcription and is cleaved post-transcriptionally to yield the mature tRNA (Figure 1b). Two Pol III transcribed yeast genes, SNR52 and RPR1,⁸ share a promoter organization similar to the proposed one, consisting of a leader sequence in which the A- and B-boxes are internal to the primary transcript but are external to the mature RNA. We thus tested whether the SNR52 and RPR1 promoter could be exploited to express *E. coli* tRNAs in yeast.

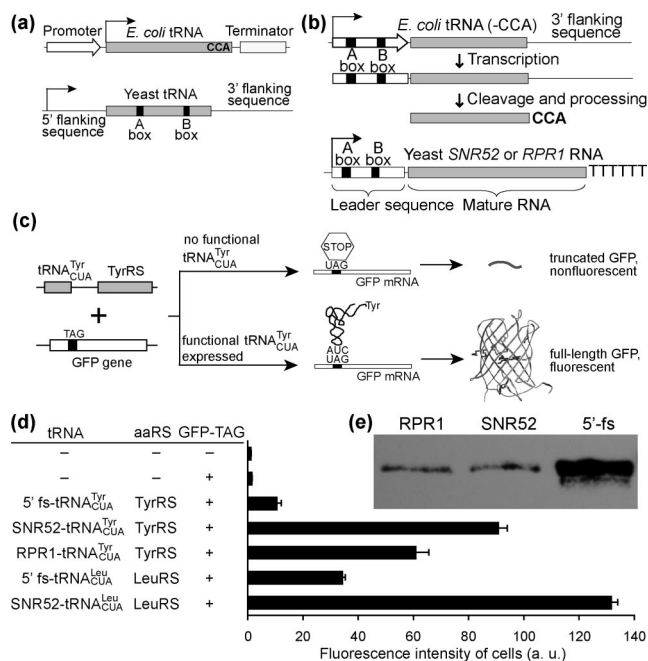


Figure 1. Efficient expression of *E. coli* tRNAs in yeast. (a) Gene elements for tRNA transcription in *E. coli* and in yeast. (b) A new method to express *E. coli* tRNAs in yeast using a Pol III promoter that contains the conserved A- and B-box and that is cleaved from the primary transcript. Gene organization of yeast SNR52 or RPR1 RNA is shown at the bottom. (c) An in vivo fluorescence assay for functional tRNA expression. (d) Fluorescence assay results for the functional expression of EctRNA_{CUA}^{Tyr} and EctRNA_{CUA}^{Leu} driven by different promoters in yeast. Error bars represent sem $n = 3$. (e) Northern analysis of EctRNA_{CUA}^{Tyr} expressed in yeast by the indicated promoters. Total RNA loaded, RPR1:SNR52:5'-fs = 10:10:1.

The gene for *E. coli* tyrosyl amber suppressor tRNA (EctRNA_{CUA}^{Tyr}) lacking the 3'-CCA trinucleotide was placed after the candidate promoter and followed by the 3'-flanking sequence of the yeast tRNA SUP4. This tRNA gene cassette was coexpressed with the cognate *E. coli* tyrosyl-tRNA synthetase (TyrRS) in *Saccharomyces cerevisiae* (Figure S1). An in vivo fluorescence assay was developed to test whether the expressed EctRNA_{CUA}^{Tyr} is functional for protein translation in yeast (Figure 1c). A TAG stop codon was introduced at a permissive site (Tyr39) of the green fluorescent protein (GFP) gene, and this mutant gene is coexpressed with the EctRNA_{CUA}^{Tyr}/TyrRS. If the EctRNA_{CUA}^{Tyr} is transcribed and correctly processed into a functional tRNA, the TyrRS will aminoacylate it with tyrosine, and the acylated EctRNA_{CUA}^{Tyr} will then suppress the TAG codon, producing full-length GFP and rendering cells fluorescent. The fluorescence intensities of cells suggest how efficiently a promoter can drive the functional expression of the EctRNA_{CUA}^{Tyr}.

in yeast. When the EctRNA^{Tyr}_{CUA} was expressed using the conventional method, the 5'-flanking sequence of an endogenous yeast tRNA SUP4, only weak fluorescence could be detected (Figure 1d). In comparison, when the EctRNA^{Tyr}_{CUA} was driven by the SNR52 or RPR1 promoter, the mean fluorescence intensities of cells were increased 9- and 6-fold, respectively. These results indicate that both the SNR52 and RPR1 promoter can drive the EctRNA^{Tyr}_{CUA} expression in yeast efficiently, and the expressed EctRNA^{Tyr}_{CUA} is functional in translation.

The transcription levels of the EctRNA^{Tyr}_{CUA} driven by different promoters were measured by Northern blot. Unexpectedly, the 5'-flanking sequence of SUP4 generated ~100-fold more EctRNA^{Tyr}_{CUA} than the SNR52 or RPR1 promoter (Figure 1e). The fact that these EctRNA^{Tyr}_{CUA} were much less active in protein translation than those expressed by the SNR52 or RPR1 promoter suggests that the EctRNA^{Tyr}_{CUA} expressed by the 5'-flanking sequence is not correctly processed or modified.

To test whether this method can be generally used to express other *E. coli* tRNAs, we replaced the EctRNA^{Tyr}_{CUA} with the *E. coli* leucyl amber suppress tRNA (EctRNA^{Leu}_{CUA}) and the TyrRS with the *E. coli* leucyl-tRNA synthetase (LeuRS). The 5'-flanking sequence of SUP4 could also drive the EctRNA^{Leu}_{CUA} expression in yeast, but the fluorescence intensity increased 4-fold when the SNR52 promoter was used (Figure 1d). According to the yeast A- and B-box identity elements,⁹ the EctRNA^{Tyr}_{CUA} does not have a fully matched A-box, while the EctRNA^{Leu}_{CUA} has matched A- and B-boxes. Regardless of the identity elements, the SNR52 promoter significantly increased the functional expression of both types of *E. coli* tRNAs in yeast.

Next we examined the effect of NMD inactivation on the UAA incorporation efficiency in yeast. The amber stop codon TAG is the most frequently used to encode UAAs, but mRNAs containing premature stop codons are rapidly degraded in yeast by the surveillant NMD pathway.⁶ We reasoned that inactivation of NMD would preserve the stability of the UAG-containing target mRNA and thus enhance the incorporation efficiency of UAAs. The yeast UPF1 gene has been shown to be essential for NMD, deletion of which restores wt decay rates to nonsense-containing mRNA transcripts.¹⁰ We therefore generated a *upf1Δ* strain of *S. cerevisiae* and compared the UAA incorporation efficiency in this strain to the wt strain.

The EctRNA^{Leu}_{CUA} driven by the SNR52 promoter and the DanRS¹¹ were used to incorporate the fluorescent UAA DanAla (Figure 2c) into the GFP at site 39. When DanAla was added to the growth media, the fluorescence intensity of the *upf1Δ* strain was doubled compared to that of the wt strain (Figure 2a). In the absence of DanAla, the intensities dropped to low background levels, suggesting high specificity of the EctRNA^{Leu}_{CUA}/DanRS pair for DanAla. We also tested the incorporation of UAA OmeTyr using the EctRNA^{Tyr}_{CUA}/OmeRS pair.⁴ When OmeTyr was added, the fluorescence intensity of the *upf1Δ* strain was also increased 2-fold compared to the wt strain. However, in the absence of OmeTyr, the fluorescence intensities in both strains were still quite high. We then expressed the EctRNA^{Tyr}_{CUA} only, without the OmeRS, and cell fluorescence intensities dropped down to the background. This result shows that the OmeRS still charges natural amino acids to the EctRNA^{Tyr}_{CUA}, consistent with the mass spectrometric analysis, in which ~7% of the incorporated amino acids were found to be natural ones.¹²

To examine how the above improvements correlate with protein yield, we expressed the GFP(39TAG) gene in the *upf1Δ*

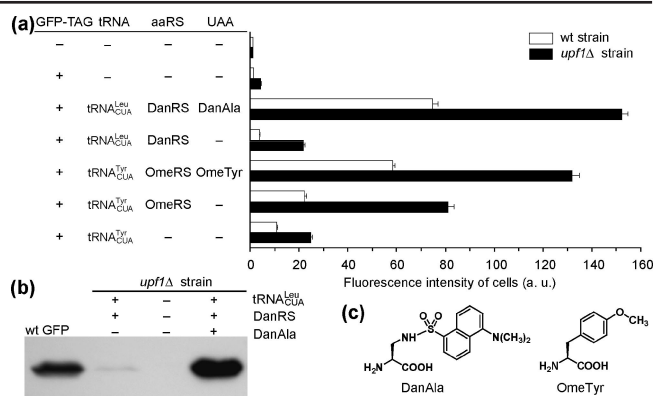


Figure 2. NMD inactivation increases the incorporation efficiency of UAAs in yeast. (a) Fluorescence assay results for UAA incorporation in wt and the *upf1Δ* strain. Error bars represent sem $n = 3$. (b) Western analysis of the DanAla-containing GFP expressed in the *upf1Δ* strain. Same amounts of cell lysate from each sample were separated by SDS-PAGE and probed with an anti-His5 antibody. (c) UAA structures.

strain using the DanRS and the EctRNA^{Leu}_{CUA} driven by the SNR52 promoter (Figure 2b). In the presence of 1 mM DanAla, the full-length GFP was produced in an overall purified yield of 15 ± 2 mg/L, ~300-fold higher than the previous system and comparable to the yield in *E. coli*.

In summary, we developed a new method for expressing orthogonal bacterial tRNA in yeast, which is general for various tRNAs and produces tRNAs highly competent in translation. We also showed that an NMD-deficient yeast strain increases the UAA incorporation efficiency. These new approaches dramatically improved the yield of UAA-containing proteins in yeast, and should be useful to similarly optimize UAA incorporation in mammalian cells. Efficient incorporation of UAAs in various live cells will be valuable to study biology through chemical means and to produce large quantities of proteins for industrial or therapeutic applications.

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Supporting Information Available: Plasmid diagrams (Figure S1); experimental materials and methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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