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Engineered rRNA Enhances the Efficiency of Selenocysteine Incorporation during Translation

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Supporting Information

ABSTRACT: We developed a new genetic selection approach to screen for mutations that can alter the efficiency of selenocysteine incorporation. We identified mutations in 16S rRNA that increase or decrease the efficiency of selenocysteine incorporation in *Escherichia coli* without influencing the efficiency or fidelity of canonical translation. Engineered ribosomes with improved selenocysteine incorporation provide valuable tools for synthetic biology and biotechnology.

Selenium is predominantly incorporated into biological systems as the unconventional amino acid selenocysteine.[1] Selenocysteine is cotranslationally incorporated into proteins in all three domains of life, providing unique biophysical properties to the resulting selenoproteins.[2,3] Incorporation of selenocysteine into proteins is unique compared to other ribosomally incorporated amino acids because selenocysteine lacks its own aminoacyl-tRNA synthetase.[4−7] Selenocysteine is synthesized in the context of a selenocysteine-specific tRNA ($tRNA^{Sec}$, encoded by the *selC* gene in prokaryotes) that recogizes the UGA stop codon.[8] The $tRNA^{Sec}$ is first misacylated with serine by the seryl-tRNA synthetase, then a selenocysteine synthase (encoded by the *selA* gene) uses selenophosphate produced by the selenophosphate synthetase (encoded by the *selD* gene) to synthesize selenocysteine directly on its tRNA (Figure 1a).[6,9−11] Insertion of selenocysteine occurs at only a few specific UGA stop codons and requires the presence of a selenocysteine insertion sequence (SECIS) in the mRNA. The SECIS is recognized by a multifunctional protein, SelB, that acts as a translation factor and recruits $tRNA^{Sec}$ to suppress the stop codon and insert selenocysteine.[12,13] The SECIS is thought to act as a proofreading element so that selenocysteine is not incorporated at all UGA codons, by activating GTP hydrolysis via SelB and increasing the local concentration of $tRNA^{Sec}$.[9,14]

Despite the detailed knowledge of the factors involved, the mechanism by which selenocysteine is incorporated during translation is not well understood. The observation that the SECIS must be precisely positioned relative to the UGA codon[15] has led to predictions that it positions the SelB−$tRNA^{Sec}$ complex in a productive orientation for entry to the ribosome.[12,16] Recent observations have shown that the ribosome itself plays a role in recognizing the aminoacylation status of incoming tRNAs,[17] modulating translation in response to structured elements in mRNAs,[18] and controlling the fidelity of decoding,[19,20] however a potential role of the ribosome in controlling the efficiency of selenocysteine incorporation has not been examined. Here we show that mutations in 16S rRNA can decrease or increase the efficiency of selenocysteine incorporation into reporter genes and endogenous *E. coli* selenoproteins. These effects were selenocysteine specific and were not a result of altered translation efficiency of the mutant ribosomes or their fidelity in canonical translation termination. We found that residues within rRNA control the balance between efficient selenocysteine incorporation and the fidelity of protein synthesis, providing new tools for biotechnology and synthetic biology.

We used an orthogonal translation system, consisting of an orthogonal ribosome and an orthogonal mRNA,[21] to link the cotranslational insertion of selenocysteine to a life−death selection in *E. coli*. We constructed a new genetic reporter that encodes a fusion between chloramphenicol acetyltransferase (CAT) and a mutant phenylalanine aminoacyl-tRNA synthetase with relaxed specificity (PheS A294G) to perform efficient positive and negative selections (*cat-pheS*).[22] Expression of CAT enables cells to survive in the presence of chloramphenicol (Cm), while production of the mutant PheS results in promiscuous incorporation of 4-chloro-DL-phenylalanine (Cl-Phe) and leads to cell death. To determine whether this reporter system could be used to examine the incorporation of selenocysteine, we inserted a SECIS derived from the *E. coli fdnG* mRNA into the *cat* gene at sites that encode surface exposed loops (Figures 1b and S1). Only insertion of the SECIS before codon 129 of *cat* enabled the production of functional CAT (Figures 1c and S2). Insertion of a UGA stop codon adjacent to the SECIS reduced the production of full length CAT dramatically, as judged by Cm resistance, consistent with the low efficiency of selenocysteine incorporation in *E. coli*.[23] Overexpression of SelA, SelB, and SelC increased the cells' resistance to Cm significantly, confirming that CAT production from the reporter was selenocysteine dependent (Figure 1c).

The 30S ribosomal subunit provides the path for mRNA as it passes through the ribosome and is responsible for decoding the mRNA using incoming aminoacyl-tRNAs and release factors.[24,25] Competition between $tRNA^{Sec}$ and release factor 2 (RF2) at the UGA codon dictates whether incorporation of selenocysteine or termination of protein synthesis occurs, respectively.[23] Therefore we reasoned that the 30S subunit might influence the efficiency of selenocysteine incorporation during translation. We used an orthogonal 16S rRNA (O-16S

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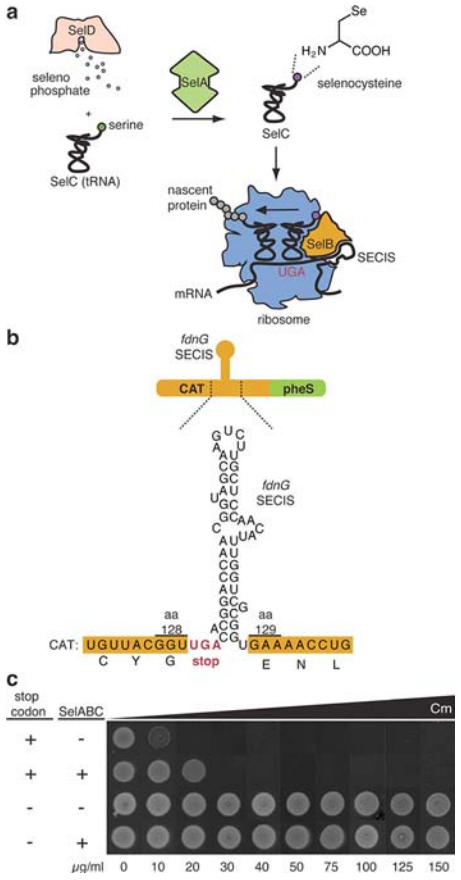

Figure 1. A selection system to analyze selenocysteine incorporation in *E. coli*. (a) Selenocysteine incorporation in prokaryotes. (b) Schematic of the reporter mRNA showing the sequence and location of the *E. coli fdnG* SECIS. (c) Cm resistance derived from a SECIS-containing reporter depends on the overexpression of SelA, SelB, and SelC.

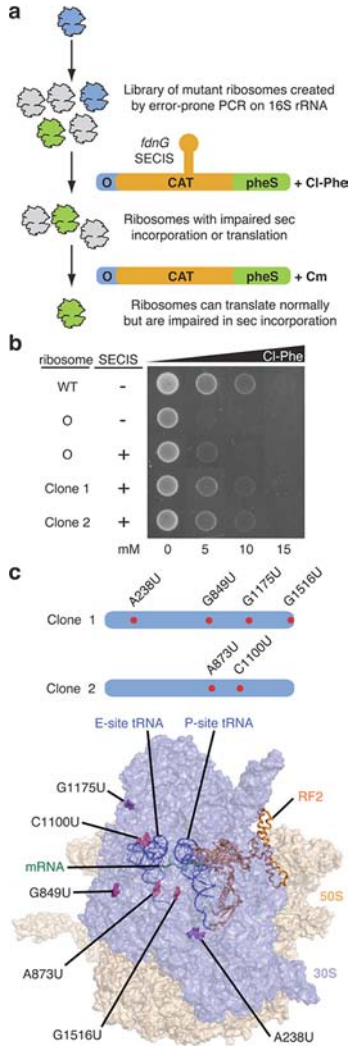

Figure 2. Selection of ribosomes with impaired selenocysteine incorporation. (a) An orthogonal translation system was used to perform a positive−negative selection to obtain ribosomes with impaired selenocysteine incorporation without compromising canonical translation. (b) Reduced sensitivity to Cl-Phe of cells expressing selected ribosomes. (c) Location of point mutations within the primary sequence of 16S rRNA and the three-dimensional structure of the ribosome (image made using Pymol, from the 3.5 Å structure of the *Thermus thermophilus* 70S ribosome in complex with mRNA, tRNAs, and RF2).[27]

rRNA) as a template for mutagenesis, as the 16S rRNA is responsible for almost all the activities of the 30S subunit and because 30S subunits formed from O-16S rRNA are not required to synthesize *E. coli* proteins and consequently the O-16S rRNA can be used for structure−function studies without lethality.[21,26] We used error-prone PCR to introduce random mutations across the full length of the O-16S rRNA and cotransformed the library of mutant O-16S rRNA expression plasmids with a *cat-pheS* reporter containing an orthogonal ribosome-binding site and a UGA-containing SECIS (O-*cat*129SECIS-*pheS*). Cells were plated on media containing Cl-Phe to select for 16S rRNA mutants that were either impaired in selenocysteine incorporation or defective in normal translation (Figure 2a). Ribosome expression plasmids were isolated from cells that survived the first selection and cotransformed with an orthogonal *cat-pheS* reporter lacking a SECIS (O-*cat-pheS*). These cells were plated on media containing Cm to remove clones that are defective in canonical translation and retain mutants that can translate normally unless SECIS-mediated UGA recoding is required. The surviving 16S rRNA clones were cotransformed with the SECIS containing O-*cat*129SECIS-*pheS* reporter and plated on media containing Cl-Phe to confirm that these clones were defective in selenocysteine incorporation (Figures 2b, S3 and S4). Sequencing of 16S rRNA expression plasmids that survived both negative and positive selections and were selectively defective in translating the SECIS-containing reporter revealed two distinct mutants (Figure 2c). Clone 1 had four point mutations in the 16S rRNA (A238U, G849U, G1175U, and G1516U), while clone 2 had two (A873U and C1100U).

To elucidate the contribution of each of the six mutations found in the clones from our selections, we made each individual mutant in the 16S rRNA as well as combinations of different mutations. To eliminate any potentially confounding effects due to the orthogonal ribosome−mRNA interaction, we made all mutants in the context of wild-type (WT) 16S rRNA (*rrnB*). 16S rRNA mutants were expressed as the sole 16S rRNAs in cells where all seven rRNA operons had been deleted.[28] All six individual mutants and most combinations could support normal cell growth (Figure S5), confirming that our selections had indeed identified mutants that did not impair canonical translation. To quantify the effect of each mutation

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on the efficiency of selenocysteine incorporation, we used a reporter mRNA where the *Gaussia princeps* luciferase (*GLuc*) was located downstream of the *fdnG* SECIS, so that luciferase activity is detected only if translation continues beyond the UGA stop codon. All of the individual mutations, with the exception of C1100U, reduced translation of the SECIS-containing reporter significantly, as did all of the combinations of mutations, with the exception of C1100U/G1516U (Figure 3a). These data indicate that these mutations reduce the ability of the ribosome to incorporate selenocysteine. Interestingly the C1100U mutation alone increased the efficiency of selenocysteine incorporation dramatically.

To quantify any possible effects of the mutations on normal translation or non-SECIS mediated UGA suppression, we selected a subset of mutants that covered the range of different efficiencies of selenocysteine incorporation. Translation of a reporter lacking a SECIS revealed that none of the mutations affected canonical translation (Figure 3b), such that the ratio of the efficiencies of selenocysteine incorporation to normal translation maintained the trends for selenocysteine incorporation alone (Figure 3c). Next we examined the effects of the 16S rRNA mutations on stop codon suppression by the well-characterized $tRNA^{Trp(UCA)}$(*trpT*) suppressor.[29] Expression of $tRNA^{Trp(UCA)}$ increased stop codon suppression significantly relative to overexpressed WT $tRNA^{Trp(CCA)}$, demonstrating that this suppressor was active in our system. None of the 16S rRNA mutants increased suppression of the UGA stop codon by $tRNA^{Trp(UCA)}$(Figure 3d and e). These data show that the effects of our selected 16S rRNA mutants on selenocysteine incorporation are not due to defects in their capacities to translate using the common 20 amino acids or to an increased efficiency of UGA stop codon suppression independent of the selenocysteine insertion machinery.

In anaerobic conditions *E. coli* expresses a selenocysteine-containing formate dehydrogenase, which can use benzyl viologen as an electron acceptor for the oxidation of formate.[30] The reduction of benzyl viologen causes this compound to change from colorless to a deep-purple color. The activity of formate dehydrogenase is strictly selenocysteine-dependent (Figure 3f, Δ*selA*) and allows the insertion of selenocysteine into an endogenous protein to be determined colorimetrically. The efficiency of selenocysteine incorporation was increased by the C1100U mutation, determined from the intensity of color generated by the reduction of benzyl viologen (Figure 3f). Interestingly the G1516U and C1100U/G1516U mutants also showed an increased selenocysteine incorporation, possibly as a result of the altered competition between RF2 and $tRNA^{Sec}$ under slow, anaerobic growth conditions.[23] These data show that the selected 16S rRNA mutations affect the efficiency of selenocysteine incorporation in *E. coli* by otherwise WT ribosomes translating an endogenous mRNA in its physiological context for selenoprotein expression.

In this study we have provided the first functional evidence for a role of rRNA in controlling the efficiency of selenocysteine incorporation in *E. coli*. The exact mechanisms involved in the effect of each individual mutation on selenocysteine incorporation cannot be resolved without structural data for the locations of the SECIS, $tRNA^{Sec}$, and SelB on the ribosome. Nevertheless, our study has identified specific residues in the 16S rRNA that are important for efficient selenocysteine incorporation in *E. coli*.

Our new genetic selection approach effectively couples cell viability in *E. coli* with the efficiency of selenocysteine incorporation in a tunable manner (SI). This approach could be applied further to examine the relative importance of each component of the selenocysteine machinery or to engineer these components to further enhance selenocysteine incorporation. We showed that the use of a second selection step using

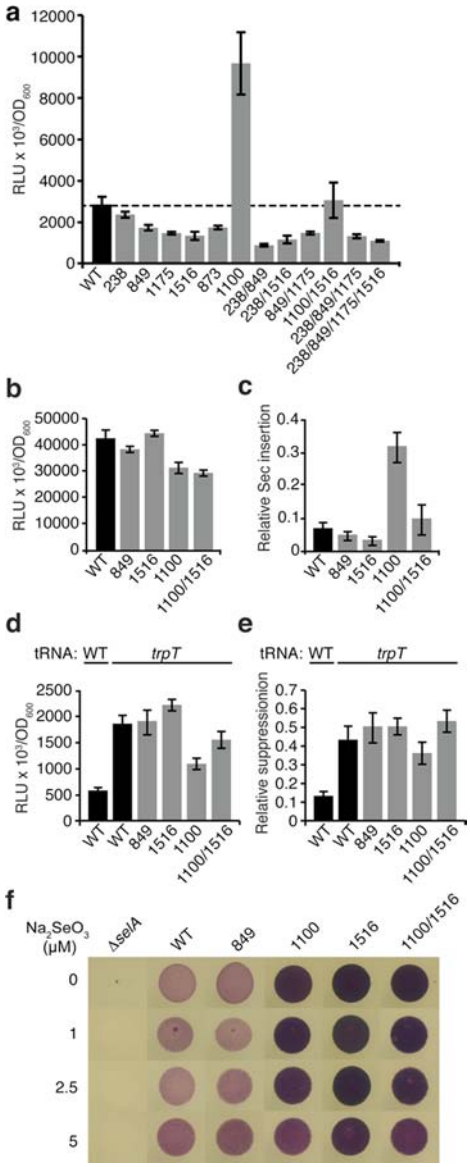

Figure 3. Mutant ribosomes with impaired or enhanced selenocysteine incorporation, independent of canonical UGA suppression. (a) Efficiency of selenocysteine incorporation by mutant ribosomes. Mutant and WT ribosomes were expressed as the sole ribosomes in cells deleted for all seven ribosomal operons and expressing a reporter mRNA with the *GLuc* located downstream of the *fdnG* SECIS. Selenocysteine incorporation was determined by measuring luciferase activity, normalized to OD_{600}. Clone 1 corresponds to mutant 238/849/1175/1516. (b) Activity of selected ribosomes in canonical translation was determined using a reporter mRNA without a SECIS. (c) Efficiency of selenocysteine incorporation by mutant ribosomes, normalized to canonical translation. (d) Effect of 16S rRNA mutations on tryptophan-mediated suppression by $tRNA^{Trp(UCA)}$. Overexpressed wild-type $tRNA^{Trp(CCA)}$ (WT) was used as a control. (e) $tRNA^{Trp(UCA)}$ (TrpT)-mediated suppression, normalized to canonical translation. (f) Selenocysteine incorporation into endogenous formate dehydrogenase is affected by mutations in 16S rRNA, determined by the reduction of benzyl viologen.[31]

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a reporter lacking a SECIS element provides a powerful means to ensure that these altered components do not interfere with canonical cellular translation. Furthermore, the tools developed here could be used for re-engineering of interactions between components, such as SelB and the SECIS, to create multiple orthogonal selenocysteine incorporation circuits in living cells. This could enable selenocysteine to be selectively incorporated into different sets of selenoproteins depending on distinct stimuli, enabling the construction of logic gates or environmental sensors based on selenoproteins.

Engineered ribosomes with an increased capacity to incorporate selenocysteine could be useful for many synthetic biology[32−34] and biotechnological applications.[35,36] For example, high-level incorporation of selenocysteine into recombinant proteins will facilitate phasing in X-ray crystallography,[35] determining disulfide bond connectivity by NMR,[37] engineering new structural motifs,[36] and the characterization and re-engineering of naturally occurring selenoproteins. Our results using suppressor tRNAs emphasize that selenocysteine incorporation is a distinct process from canonical nonsense suppression. The use of this system as a scaffold to expand the genetic code with unnatural amino acids could provide advantages over currently used amber suppression technologies that result in promiscuous insertion of unnatural amino acids at all UAG codons.[38−40] In addition, the unique mechanism by which selenocysteine is synthesized could enable the genetic code to be expanded with reactive amino acids that cannot be incorporated using current approaches.[38−40] Selenocysteine is synthesized on its tRNA to avoid the severe consequences of toxicity from free selenocysteine. Since we show that the efficiency of incorporation can be increased using artificially evolved ribosomes, it could be feasible to further engineer selenocysteine incorporation machinery to add highly reactive amino acids to the genetic codes of living cells.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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