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Ribosome Evolution for Two Artificial Amino Acids in *E. coli*

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Specific incorporation of artificial amino acids into polypeptides offers a useful tool for studying and expanding function. Neumann et al. (2010) recently described the evolution of an *E. coli* ribosome that incorporates two artificial amino acids at two designated sites into proteins, in combination with amber codon and a four-base codon.

Expansion of the genetic code has revolutionized the translation machinery-based use of biotechnology for protein or polypeptide expression in which artificial (unnatural) amino acids can be included into the synthesized polypeptide chain (Cornish et al., 1995). The team of Peter G. Schultz has been making a major contribution to the development of such an approach, where an orthogonal pair of tRNA and aminoacyl-tRNA synthetase (ARS) functions in vivo to generate an aminoacyl-tRNA charged with an artificial amino acid (Xie and Schultz, 2006). The amber stop codon, UAG, is generally used for the assignment of an artificial amino acid, since this nonsense codon is most readily suppressible by a tRNA-bearing CUA anticodon ($tRNA_{CUA}$) competing with release factor 1 (RF1) in *E. coli* or other translation systems. Despite the fact that a few dozen of the orthogonal tRNA-ARS pairs are available to date, the expression of desired proteins under the suppression of amber codon occasionally suffers from poor expression efficiency, due to the competing translation termination by RF1 that depends on the incorporation site (e.g., the neighboring codons) or the structure of artificial amino acids. Moreover, the amber suppression strategy is limited to the incorporation of a single type of artificial amino acids into one protein at a time.

The team of Jason W. Chin has made tremendous efforts to evolve 16S ribosomal RNA (rRNA) from the small subunit of *E. coli* ribosome over the last half decade, attempting to circumvent the above restrictions. As a first step, they isolated ribosome variants that recognize a mutant Shine-Dalgano (SD) sequence of mRNA by means of the genetic selection, leading to the development of an orthogonal pair of ribosome and mRNA (O-ribosome and O-mRNA) (Rackham and Chin, 2005). The virtue of developing the O-ribosome—O-mRNA pair is that it facilitates evolution of O-ribosome. Since the wild-type (WT) ribosome and O-ribosome can coexist in *E. coli*, and the O-ribosome does not translate natural mRNAs with the WT SD sequence, even toxic O-ribosome mutants can be evolved without suffering from the fatal outcome. In fact, in their follow-up work, a new O-ribosome mutant, referred to as Ribo-X, was identified and shown to have a unique function, as RF1 was less efficient in termination of the amber codon, and thereby the amber codon was suppressed by aminoacyl-$tRNA_{CUA}$ more effectively (Wang et al., 2007). This notable work developing Ribo-X has circumvented one of the intrinsic restrictions that originates from the competition with RF1 on the amber codon.

To overcome the remaining limitation described above, it is required to evolve a new mutant ribosome that is able to use another suppression codon for the incorporation of the second artificial amino acid. Fortunately, such a codon, the so-called four-base codon, inducing +1 frameshift to incorporate an artificial amino acids was already available (Hohsaka et al., 1996); it was even demonstrated that two artificial amino acids were incorporated into the desired specific sites in the combination of amber codon and a four-base codon using an *E. coli* in vitro translation system (Murakami et al., 2003). The team of Chin ingeniously generated a library 16S rRNA mutants based on Ribo-X that focuses on the region of the decoding center, and selected those capable of decoding a four-base codon by the use of aminoacyl-tRNA that had the corresponding anticodon (Neumann et al., 2010). One of the ribosome mutants, referred to as Ribo-Q, was able to effectively read a four-base codon, such as AGGA, UAGA, AAGA, or CCCU, and incorporate an artificial amino acid into a nascent polypeptide chain at one of these codons in *E. coli*. Moreover, the double suppression of AGGA and UAG codons with *p*-azido-L-phenylalanine and N6-[(2-propynyloxy)-carbonyl]-L-lysine paired with the available mutants of MjTyrRS and MbPylRS, respectively, was demonstrated. This remarkable work gives us a new opportunity to explore the technical merits of expressing proteins that contain two or possibly more artificial amino acids in vivo.

Although the decoding efficiency of four-base codon by Ribo-Q was significantly increased compared with that by the WT ribosome, it suffered from the production of a truncated protein as a by-product, due to a failure of the desired frameshift (as shown in Figure S6, S7, and S13 in Neumann et al., 2010). Why? The concrete mechanistic reason for this must wait for the structural analysis of the individual complexes of Ribo-Q with four-base anticodon tRNAs, but the recent X-ray analysis of WT ribosome complexed with some four-base anticodon tRNAs at A site has suggested that the anticodon unlikely forms simple four base pairs with the four-base codon on mRNA (Dunham et al., 2007). When the four-base anticodon tRNA binds to the A site of ribosome, the first base (at position 33.5) in the four-base anticodon possibly unpaired with the forth base in

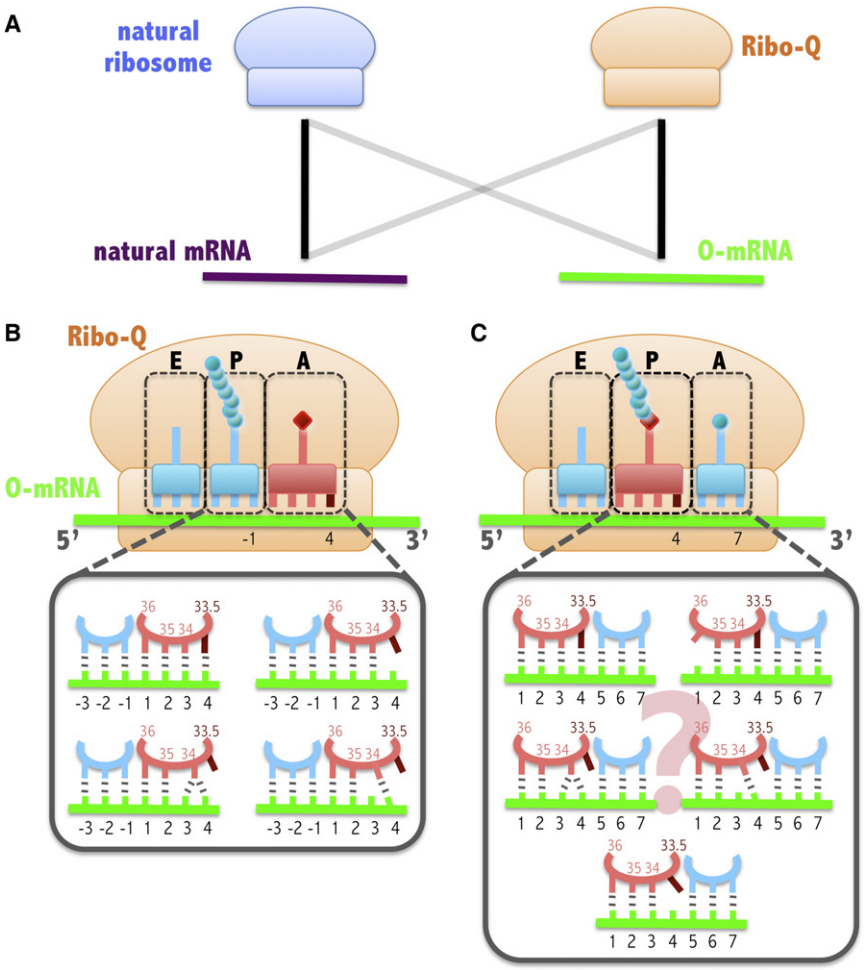

Figure 1. Decoding an mRNA Four-Base Codon in a Ribosome
(A) Orthogonal relationship between wild-type ribosome-mRNA and orthogonal ribosome-mRNA.
(B) Decoding four-base codon in the A site.
(C) Decoding four-base codon in the P site. There are multiple possibilities of the decoding mode depending upon the sequences of four-base codon and neighboring codons. Numbers colored in red or black represent numbering of anticodon or mRNA bases, respectively. An expanded base on a quadruplet anticodon is indicated as 33.5 in dark red.

the four-base codon (Figure 1B); alternatively, the second base (at position 34) in the four-base anticodon may sit in the flanking region between the third and forth bases in the four-base codon or even pair with the forth base (Figure 1B). When the four-base anticodon tRNA is translocated to the P site of ribosome after peptidyl-transfer, the frameshift may occur via several possible ways under similar pairing modes albeit with no structural evidence at this time (Figure 1C). This complexity may be further influenced by the neighboring codon (position 5–7) that must be decoded in the designated frame (i.e., after the programmed frameshift). The first three nucleotides of four-base codons are designed by the use of rare codons, such as AGG and CCC, in *E. coli* in order to be efficiently suppressed by the four-base anticodon tRNA rather than naturally occurring tRNAs with three-base anticodon. Thus, the choice of such rare codons limits the creation of four-base codons along with satisfactory suppression efficiencies.

Some challenges still remain for the future exploration to achieve an ultimate goal of protein expression using more than two artificial amino acids in *E. coli*. However, there is no doubt that the successful evolution of Ribo-Q using the orthogonal ribosome-mRNA strategy is a big step toward developing a technology that expresses proteins with multiple artificial amino acids, leading to a new generation of biotechnology.

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