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Upgrading aminoacyl-tRNA synthetases for genetic code expansion

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Synthesis of proteins with non-canonical amino acids via genetic code expansion is at the forefront of synthetic biology. Progress in this field has enabled site-specific incorporation of over 200 chemically and structurally diverse amino acids into proteins in an increasing number of organisms. This has been facilitated by our ability to repurpose aminoacyl-tRNA synthetases to attach non-canonical amino acids to engineered tRNAs. Current efforts in the field focus on overcoming existing limitations to the simultaneous incorporation of multiple non-canonical amino acids or amino acids that differ from the L-α-amino acid structure (e.g. D-amino acid or β-amino acid). Here, we summarize the progress and challenges in developing more selective and efficient aminoacyl-tRNA synthetases for genetic code expansion.

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Introduction

All living organisms synthesize proteins using the same set of twenty canonical amino acids (AAs) according to the rules of the genetic code. Over the past two decades, efforts to change these rules have resulted in the co-translational incorporation of numerous structurally diverse non-canonical AAs (ncAAs) into proteins by codon reassignment. Genetic code expansion (GCE) is now possible in a wide range of model organisms, which has facilitated investigation of protein structure and function in various biological processes, as well as development of therapeutics and the biocontainment of genetically modified organisms.

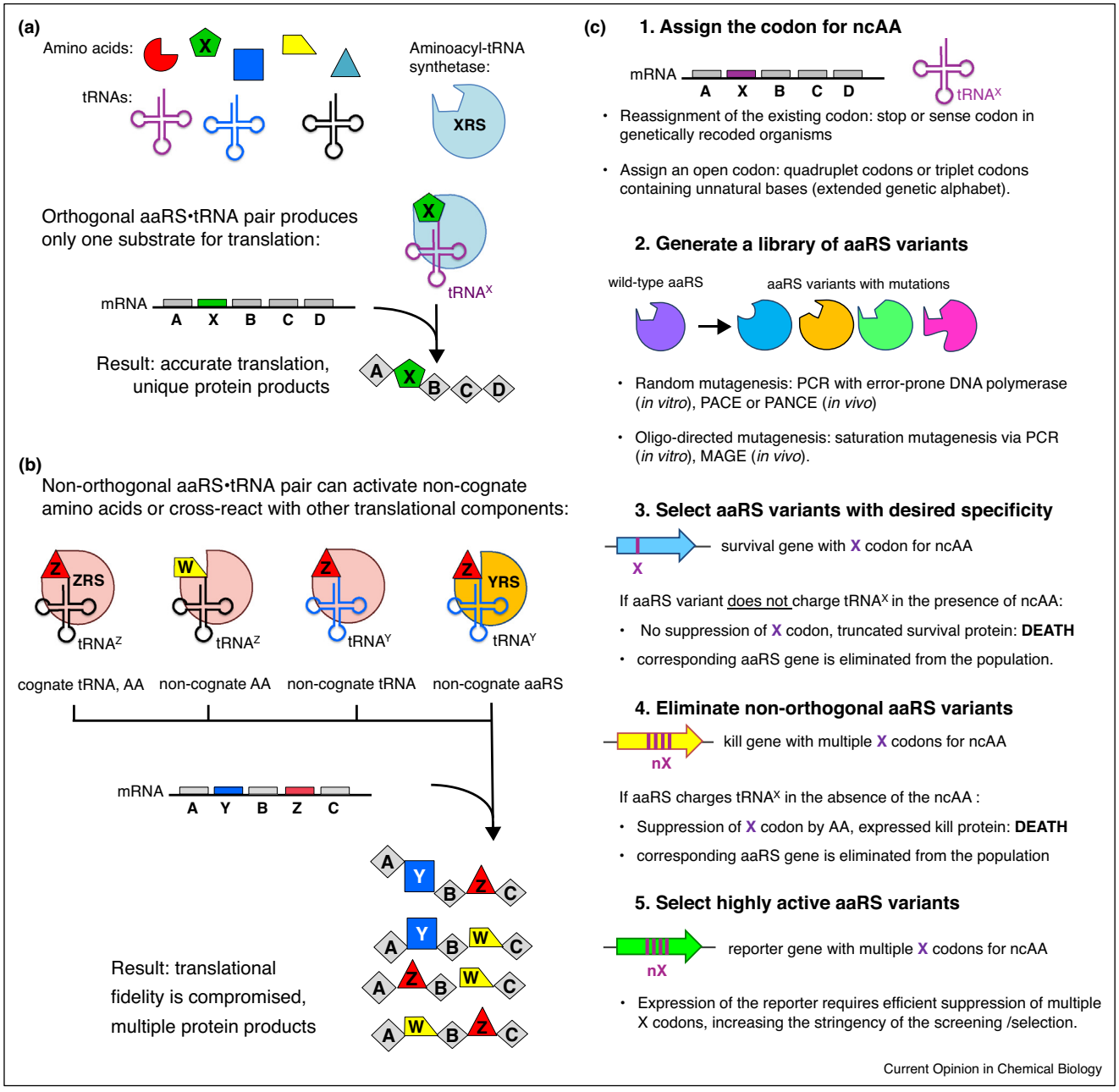
Expanding the genetic code entails altering two fundamental steps in translation: synthesis of aminoacyl-tRNAs (AA-tRNAs), and codon-anticodon pairing in the ribosome [1••]. This is achieved by repurposing an aminoacyl-tRNA synthetase (aaRS) to ligate the desired ncAA to a dedicated tRNA capable of inserting the ncAA at a defined codon (Figure 1a). For correct *in vivo* incorporation of ncAAs, engineered aaRS•tRNA pairs should not cross-react with the host aaRSs and tRNAs (*i.e.* they should be orthogonal), a requirement often met by relying on aaRS•tRNA pairs from organisms belonging to different domains of life.

Although over 200 ncAAs have been incorporated into proteins, engineered orthogonal aaRSs (o-aaRSs) usually have lower catalytic activity than their parental aaRSs, yet they maintain their AA polyspecificity (Figure 1b). These characteristics have hampered the development of more sophisticated applications (*e.g.* mass production of ncAA-containing proteins, multi-site incorporation of an ncAA, simultaneous incorporation of various ncAAs into a protein, or sense codon reassignment). Here we review fundamental aspects of aaRS specificity and efficiency and discuss recent advances in the development and discovery of superior engineered aaRSs that can contribute to overcoming the current limitations of GCE.

Fundamentals of aaRS substrate specificity

Each canonical AA has its cognate aaRS. This ensures accurate genetic code interpretation by specific pairing of AAs with tRNAs with the correct anticodon (Figure 1a). aaRSs select their cognate tRNAs through a defined set of nucleotides known as identity elements [2]. The majority of these elements are located in the anticodon loop and the acceptor stem of the tRNA, but the specific positions are unique to each tRNA for a particular AA [2]. Specific interactions with the anticodon are characteristically absent in some aaRSs, such as pyrrolysyl-tRNA synthetase (PylRS), seryl-tRNA synthetase (SerRS), or leucyl-tRNA synthetase (LeuRS), making their corresponding tRNAs well-suited for codon reassignment (Figure 1c) [2,3]. tRNA identity elements are not fully conserved throughout evolution for many aaRS•tRNA pairs, which — in some cases — prevents cross-reaction between aaRSs and tRNAs from a different species. The phylogenetic diversity of tRNA identity elements for an AA underlies the principle of orthogonality that enables GCE *in vivo*.

Figure 1



Accurate protein synthesis and conventional methodology to alter aaRS specificity. **(a)** Each aaRS (XRS) must selectively attach a cognate AA (denoted as X) to a dedicated tRNA with the correct anticodon to form aminoacyl-tRNA (X-tRNA^X). Correct codon-anticodon matching in the ribosome ensures accurate incorporation of X at a defined position in a protein sequence. **(b)** Polyspecific aaRSs recognize more than one amino acid (depicted by YRS and ZRS) or more than one tRNA (ZRS). These errors in aminoacylation result in expression of a gene into proteins with varied primary structures. **(c)** General steps for directed evolution of aaRSs.

Because AAs provide a less diverse set of structural and chemical features to help ensure accurate substrate selection, many aaRSs occasionally produce noncognate AA-tRNAs (Figure 1b). Despite detrimental consequences associated with mistranslation [4], organisms can tolerate errors in their proteomes [5] (Figure 1b). This is commonly exploited by using wild-type methionyl-tRNA synthetase to randomly incorporate selenomethionine or azidohomoalanine for X-ray crystallography applications [6,7] or *in vivo* protein labeling [8••]. However, site-specific incorporation requires development of an aaRS•tRNA pair dedicated to the particular ncAA.

AaRS•tRNA pairs for GCE applications

Over two-thirds of all ncAAs incorporated to date are mediated by the wild-type or engineered *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase (TyrRS)•$tRNA^{Tyr}$ pair or the *Methanosarcina* PylRS•$tRNA^{Pyl}$ pair (Table 1). These pairs are now standard tools for GCE applications because of their robust orthogonality in *Escherichia coli* and the tolerance of both aaRSs to anticodon mutations in their tRNAs. These features simplify the engineering of PylRS and TyrRS for a desired ncAA by *in vivo* selection methods (Figure 1c) and facilitate the targeting of different codons for reassignment (Table 1). The *M. jannaschii* TyrRS•$tRNA^{Tyr}$ pair is used in bacteria such as *E. coli* [1••], *Salmonella* [9], *Streptomyces* [10], and *Mycobacterium* [11] (Table 1); yet this pair is not suitable for eukaryotic applications because identity elements overlap with eukaryotic TyrRS•$tRNA^{Tyr}$. However, the *E. coli* TyrRS•$tRNA^{Tyr}$ pair has been adapted for GCE in diverse eukaryotic species [12]; although, the repertoire of ncAA substrates is smaller (Table 1). In contrast to the TyrRS system, the *Methanosarcina* PylRS•$tRNA^{Pyl}$ pairs are orthogonal to a much wider range of hosts, including higher eukaryotes, due to the distinctive structure of $tRNA^{Pyl}$ and its unique mode of recognition by PylRS [3,13•]. Several other o-aaRS•tRNA pairs have been developed for GCE applications, but poor orthogonality and/or limited ncAA specificity restrict their use to only a few hosts (Table 1).

Efficiency of o-aaRS•tRNA pairs

The lower catalytic activity of evolved o-aaRSs results in modest concentrations of ncAA-tRNAs. This impedes successful incorporation of ncAAs into multiple positions within one protein, and prevents efficient competition with endogenous AA-tRNAs for reassignment of sense codons. High-quality libraries of diverse aaRS variants are paramount to obtaining highly active and ncAA-specific aaRSs (Figure 1c). Oligo-directed libraries depend on the availability of high-resolution crystal structures of aaRSs [14], as well as docking studies to identify active site residues that may contact the ncAA [15,16]. In practice, the size of site saturated libraries that can be subjected to comprehensive analysis with standard lab resources is limited to 10^9, which corresponds to 6 residues

Table 1

Orthogonal aaRS•tRNA pairs applied for genetic code expansion

	Source	Orthogonal hosts	ncAA substrates[a,b]	Reassigned codons[a]	Mutual orthogonality
TyrRS	*Methanocaldococcus jannaschii*	Bacteria[a]	>50 Tyr analogs	UAG, UGA, AGGA, UAGA	PylRS, SepRS
TyrRS	*Escherichia coli*	Yeasts[a], *Caenorhabditis elegans* [70], *Danio rerio*, *Mus musculus* [71], mammalian cell lines[a]	mY, pPpa, pMpa, AzF, AcF, BnzylF, pIF	UAG	N.D.
PylRS	*Methanosarcina barkeri or mazei*	Bacteria[a], *Mus musculus* [72], *Danio rerio* [71], *Saccharomyces cerevisiae* [73,74], *Arabidopsis thaliana* [75], *Drosophila melanogaster* [74], mammalian cell lines[a]	>100 diverse AA	UAA, UAG, UGA, AGU	MjTyrRS, EcLeuRS, EcTrpRS
LeuRS	*Escherichia coli*	mammalian cell lines [76], *Saccharomyces cerevisiae* [77], *Caenorhabditis elegans* [70]	DanAla, 2-aminocaprylic acid, mY, nbC, dMnbS	UAG	PylRS
TrpRS	*Escherichia coli*	Human cell lines [78]	5PrW, 5HTP, 5AzW, 5AmW	UAG	N.D.
TrpRS	*Saccharomyces cerevisiae*	*Escherichia coli* [79,80]	NapAla, 1MeW, BtAla, MeW	UAG	N.D.
SepRS	*Methanocaldococcus jannaschii*	*Escherichia coli* [25]	Sep, pThr	UAG	PylRS, MjTyrRS
LysRS	*Pyrococcus horikoshii*	*Escherichia coli* [48]	Homoglutamine	AGGA	MjTyrRS
PheRS	*Saccharomyces cerevisiae*	*Escherichia coli* [77,81]	pFF, 2Nal, pBrF	UAG, UUU, UUG	N.D.

[a] For a full list see reference [1••].

[b] For a comprehensive list see [1••,34•]. N.D., not determined. MjTyrRS, TyrRS from *M. jannaschii*; EcLeuRS, LeuRS from *E. coli*; EcTrpRS, TrpRS from *E. coli.* mY, *p*-methyl-Tyr; AcF, *p*-acetyl-Phe; BnzylF, *p*-benzoyl-Phe; AzF, *p*-azido-Phe; pIF, *p*-iodo-Phe; pPpa, propargyloxy-Phe; pMpa, methoxy-Phe; DanAla, 2-amino-3-(5-(dimethylamino) naphthalene-1-sulfonamido) propanoic acid; nbC, *O*-nitrobenzyl-cysteine; dMnbS, 4,5-dimethoxy-2-nitrobenzyl-L-serine; 5PrW, 5-(2-Propyn-1-yloxy)-L-tryptophan; 5HTP, 5-hydroxy-L-tryptophan; 5AzW, 5-azido-L-tryptophan; 5AmW, 5-amino-L-tryptophan; Sep, *O*-phospho-L-serine; pThr, *O*-phospho-L-threonine; NapAla, 3-(1-naphthyl)-L-alanine; 1MeW, 1-methyl-L-tryptophan; BtAla, 3-benzothienyl-L-alanine; 6MeW, 6-methyl-L-tryptophan; pFF, *p*-fluoro-Phe; 2Nal, 2-naphthylalanine; pBrF, *p*-bromo-Phe.

Box 1 Cellular factors that influence the artificial evolution of orthogonal aaRSs

- Expression levels of the o-aaRS•tRNA pair

Fine-tuning the expression levels of the o-aaRS•tRNA pairs is essential as overexpression can be detrimental for cell sustainability and/or increase cross-reaction with the host aaRSs and tRNAs. Conversely, low expression of the orthogonal pair may lead to a false negative result (too low expression of the selection reporter).

- Intracellular concentration of the ncAA

The intracellular concentration of many ncAAs is limited by host cell permeability, which increases the stringency of the selection. To overcome this, ncAAs can be delivered as dipeptides [66] or, alternatively, can be synthesized *in situ* synthesis through metabolic engineering [33••].

- Orthogonality of the ncAA

Many ncAAs may be substrates of host aaRSs, which can lead to proteome-wide incorporation of the ncAA [28]. Moreover, ncAAs may interfere with essential metabolic pathways or be metabolized into a different substrate [43].

- Translation factors and quality control mechanisms

Several AA-tRNA deacylases have been recently shown to hydrolyze ncAA-tRNAs [67,68]. The delivery of ncAA-o-tRNA to the ribosome may be impaired by weak binding with elongation factor Tu [25]. Additionally, incorporation of the ncAA into the nascent protein may be limited by the ribosome [45]. Both of these issues may require further engineering of the elongation factor and the ribosome.

- Sensitivity of the selection reporter

Most selection reporters do not depend on the identity of the incorporated AA. Thus, canonical AA mischarging by o-aaRS variants [69] and suppressor tRNAs that are not fully orthogonal obscure results. The latter may abolish the possibility of negative selection [33••].

randomized using redundant NNK primers [16]. Proficient o-aaRS variants are identified by selection or screening techniques, typically using reporter genes that confer antibiotic resistance or encode fluorescent proteins (Figure 1c). However, robust and stringent phenotypes that unequivocally signal the incorporation of the ncAA are rare. Furthermore, most phenotype-based selections are sensitive to other factors (e.g. the protein expression level or relative cellular solubility of o-aaRS mutants) that can obstruct the selection of genuinely specific variants (Box 1). Despite the limitations imposed by conventional methods of mutagenesis and selection [17••], many ncAA-charging aaRS variants have been evolved and ncAA-containing proteins produced (Table 1). Nonetheless, innovative methods have been recently applied to the evolution of o-aaRSs that enabled engineering of more active and selective aaRSs.

Multiplex automated genome engineering (MAGE) facilitates the creation of combinatorial genetic diversity within a cell population by simultaneously mutagenizing different loci [18] (Figure 2a). With MAGE, a large library of chromosomally integrated *M. jannaschii* TyrRS variants was created with up to 12 mutational changes in the active site and anticodon binding domains [19••]. Genomic integration of the TyrRS variant genes increased selection stringency and prevented gene dosage fluctuations linked to plasmid-based expression. TyrRS variants generated by MAGE were capable of mediating specific ncAA incorporation in response to 30 UAG codons of a reporter gene after a single selection step and two rounds of cell sorting [19••].

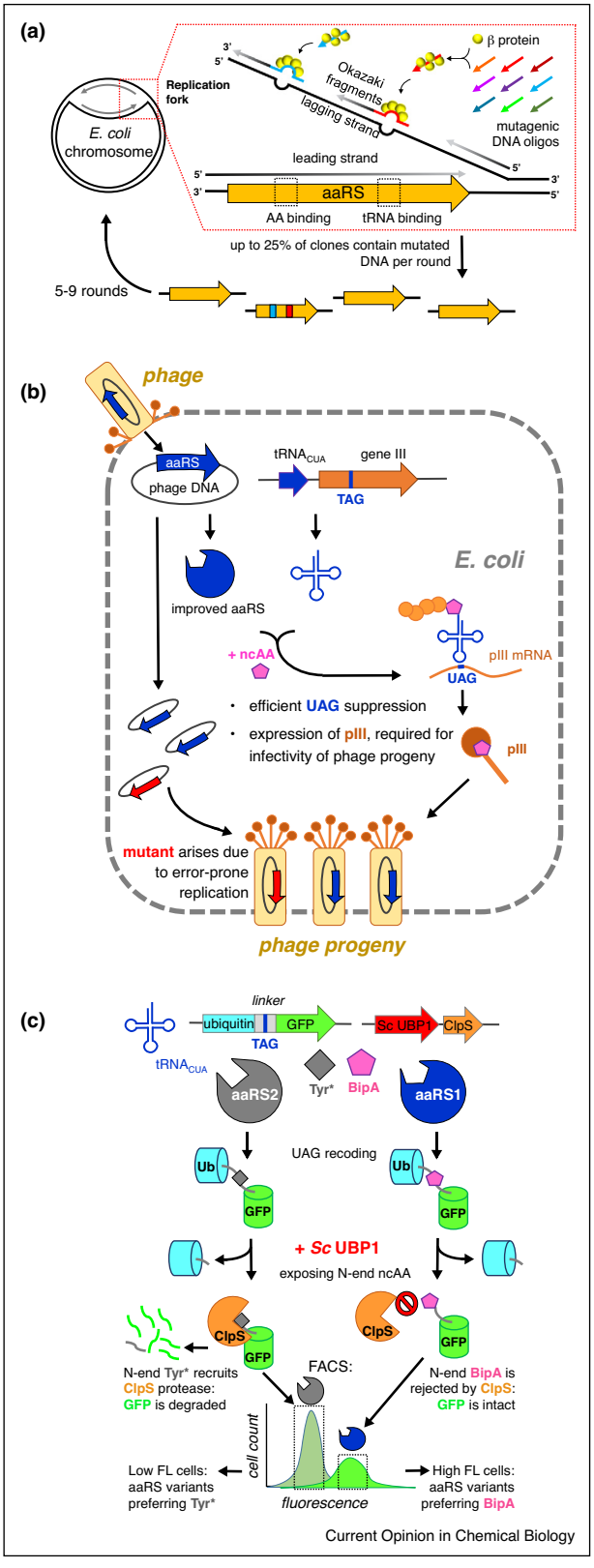
Phage-assisted continuous evolution (PACE) has emerged as powerful technology that links bacteriophage propagation with the evolution of a single gene expressed in *E. coli* [20] (Figure 1c). PACE employs a plasmid-based mutagenesis system that enables creation of random libraries with a defined mutational frequency [21]. Thus, by coupling mutational and selection cycles, PACE allows ncAA-specific aaRSs to emerge after only 48 hours or highly active variants after 20 days [22••] (Figure 2b). The latter was demonstrated by the evolution of a chimeric PylRS variant with 45-fold higher activity toward Boc-lysine [22••]. Similar results were obtained using phage-assisted noncontinuous evolution (PANCE) [13•]. This adaptation of PACE relies on serial flask transfers instead of a continuous flow machine although highly active variants require more time to emerge [13•]. Importantly, PACE and PANCE do not rely on prior structural or functional knowledge, like oligo-based mutagenesis methods do. Thus, while rational design assisted by computational methods [15] may help identify proficient aaRS variants harboring mutations outside the AA binding site [23•], approaches for random mutagenesis *in vivo* may reveal otherwise unpredictable effects of residues governing o-aaRS activity.

tRNA engineering can also improve the efficiency of an o-aaRS•tRNA pair by optimizing orthogonality of the tRNA, its binding to the o-aaRS or elongation factor Tu, or the decoding strength of the targeted codon [24–29] (Box 1). Also, the role of heterologous tRNA post-transcriptional modifications is emerging as a considerable factor in improving o-tRNA proficiency [30–32]. In cases when poor tRNA orthogonality impedes negative selection (Figure 1c), parallel positive selection schemes may be developed [33••]. With the advances in deep sequencing techniques, the most abundant members of the resulting population can be identified as potential gain-of-function variants.

Polyspecificity of o-aaRS•tRNA pairs

As described above, many aaRSs are polyspecific (i.e. they can charge multiple ncAAs), which can be exploited to incorporate ncAAs without altering the active site of the enzyme. Wild-type PylRS has a remarkably large AA binding site that accommodates >20 lysine derivatives [34•] (Table 1). This inherent polyspecificity is also displayed by engineered o-aaRSs. For example, one engineered PylRS mutant was shown to acylate at least

Figure 2



Advanced methodology for aaRS evolution. **(a)** Multiplex-automated genome engineering (MAGE) [18,19••] uses a modified λ-Red allelic replacement system to introduce changes in a targeted chromosomal gene (e.g. orthogonal aaRS) by integrating mutagenic oligos. Short regions flanking the randomized sequence of the oligo direct β protein to introduce the primer into the replication fork. After two rounds of duplication, only one of the DNA copies from the depicted replication fork will incorporate mutagenic oligos, resulting in the production of no more than 25% of recombinant clones for every round of MAGE. The desired level of diversity is achieved by repeating MAGE cycles, increasing the size of the oligo library, and the number of targeted sites. **(b)** Phage-assisted continuous evolution (PACE) [21,22••] employs a bacteriophage that lacks the gene for protein III (pIII), which is required for infectivity. A copy of the pIII gene, with at least one TAG codon, is encoded in a plasmid in *E. coli*. Active aaRS mutants aminoacylate the suppressor tRNA leading to pIII expression and propagation of bacteriophage. **(c)** Post-translational proofreading (PTP) [39•] exploits the N-end rule for protein degradation in *E. coli*. Green fluorescent protein (GFP) is fused to ubiquitin (Ub), separated by a TAG stop codon. Expression of the full-length reporter is the result of aminoacylation of a suppressor tRNA by orthogonal aaRS variants. Ubiquitin cleavase protein 1 (UBP1) from *S. cerevisiae* cleaves Ub from the reporter, exposing the N-terminus residue of GFP. ClpS surveys the N-terminus of GFP and applies the N-end rule to facilitate degradation of GFP if an undesired AA is incorporated. In contrast, if the desired ncAA is installed at the N-terminus, GFP accumulates and cells with high fluorescence emission are sorted and collected. The system is adaptable as ClpS can be engineered to discriminate against specific ncAAs.

15 different phenylalanine (Phe) analogs [35]. The ncAA polyspecificity of both wild-type and evolved aaRSs may be caused by the lack of exposure to non-cognate substrates in the course of their evolution. In some cases, the ability of o-aaRS to use structurally similar ncAAs can be advantageous [36–38]. In other cases it can hamper unambiguous site-specific incorporation of multiple ncAAs into a single protein (see below).

Using PACE with a round of negative selection, a *M. jannaschii* TyrRS variant that acylates Phe, *p*-nitro-Phe, and iodo-Phe was evolved to increase its selectivity for iodo-Phe >23 fold [22••]. An alternative approach for screening more selective aaRSs was recently devised (Figure 2c) [39•]. This method hijacks a natural protein degradation pathway in *E. coli* to screen for *M. jannaschii* TyrRS variants capable of selectively charging the ncAA biphenylalanine (BipA) and discriminating against other Tyr analogs that were substrates of the progenitor TyrRS. By supplying undesired ncAA substrates during the rounds of negative screening, a TyrRS variant emerged with a much narrower substrate selectivity for BipA. Surprisingly, similar to the PACE-evolved PylRS variants with improved activity, the resulting mutations in *M. jannaschii* TyrRS were located outside of the active site region.

Acylation of amino acids with non-canonical backbones

Recent characterization and engineering of translation apparatus components have shown that genetic incorporation of ncAAs with structures beyond the L-α structure is possible *in vitro* [40•,41]. These efforts have relied on

the synthesis of ncAA-tRNAs using alternative *in vitro* aminoacylation techniques such as flexizyme [42]. Although wild-type aaRSs were known to acylate L-β-AAs and D-α-AAs [43,44] and can facilitate the incorporation L-β-Phe *in vivo* [45], the recombinant protein yield was low. The progress in evolving aaRSs for these AAs will depend on engineered ribosomes that effectively catalyze peptide bond formation between AAs with altered backbones [45,46].

Codon reassignment and development of mutually orthogonal o-aaRS•tRNA pairs

One of the current challenges in GCE is the incorporation of multiple different ncAAs into a single protein. This task faces two difficulties: the need for mutually orthogonal o-aaRS•tRNA pairs (Table 1, [47–49]) and emancipation of more codons that can be reassigned with ncAAs. While UAG-codon and rare sense codon recoded strains [50–53] improve the incorporation of individual ncAAs in response to reassigned codons, additional efforts are needed to make these strains amenable to multiple ncAA installment. ncAA incorporation in response to quadruplet codons capitalizes on the ability of natural translational machinery to tolerate frameshift suppression [54] but requires ribosome adaptation [55] and proficient o-tRNA variants [27]. Emerging techniques to rapidly liberate sense codons [56,57] highlight the need for deeper understanding of viable codon replacements [58] due to the inherent influence of codon usage on various cellular processes, including translation [59].

Organisms with 6-letter genetic alphabets provide an alternative to genomically recoded organisms (Figure 1c) [60]. tRNAs containing unnatural bases within the anticodons can decode cognate unnatural codons and direct site-specific incorporation of ncAAs [61••]. Importantly, the anticodon-recognizing aaRSs can tolerate these unnatural bases within the anticodon [61••,62]. It remains unknown if tRNAs with unnatural bases can achieve full orthogonality.

Outlook

Discovery of new orthogonal aaRS•tRNA pairs through bioinformatics analyses combined with innovative techniques for directed evolution allows selection of o-aaRSs variants with high catalytic efficiency and specificity. This will advance the application of orthogonal translation systems in cutting-edge research, such as biocontainment of synthetic organisms with rewired genomes [53,57,63] or expanded genetic alphabets [61••], production of avirulent, yet fully infectious viruses for effective vaccines [64], development of stable delivery vehicles for gene therapy [65], or a host of still unknown applications.

Conflict of interest statement

Nothing declared.

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