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Recent advances in genetic code engineering in *Escherichia coli*

Michael Georg Hoesl and Nediljko Budisa

The expansion of the genetic code is gradually becoming a core discipline in Synthetic Biology. It offers the best possible platform for the transfer of numerous chemical reactions and processes from the chemical synthetic laboratory into the biochemistry of living cells. The incorporation of biologically occurring or chemically synthesized non-canonical amino acids into recombinant proteins and even proteomes via reprogrammed protein translation is in the heart of these efforts. Orthogonal pairs consisting of aminoacyl-tRNA synthetase and its cognate tRNA proved to be a general tool for the assignment of certain codons of the genetic code with a maximum degree of chemical liberty. Here, we highlight recent developments that should provide a solid basis for the development of generalist tools enabling a controlled variation of chemical composition in proteins and even proteomes. This will take place in the frame of a greatly expanded genetic code with emancipated codons liberated from the current function or with totally new coding units.

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Introduction

Nature builds up proteins with the 20 canonical amino acids (cAAs) encoded by the 61 sense codons. However, these 20 side-chain functionalities are obviously not sufficient for proteins to cover all the chemical diversity necessary to maintain many vital biological functions in both unicellular and multicellular organisms. Evolution invented two strategies to increase the side-chain inventory: a small fraction of proteins is co-translationally equipped with special proteinogenic amino acids such as selenocysteine (Sec) and pyrrolysine (Pyl) by reassignment of termination codons. However, the major classes of chemical modifications that contribute to the protein structure/function diversity are post-translational modifications (PTMs). These reactions are selectively and timely coordinated chemistries performed by dedicated enzymes and enzymatic complexes, usually in specialized cell compartments.

Certainly, one of the main goals of Synthetic Biology is to generate new and emergent biological functions in streamlined cells which are equipped with 'tailor-made biochemical production lines'. However, it is extremely difficult to mimic nature's complex machineries such as the PTM-apparatus. Thus, we usually highjack and/or divert cellular systems such as protein translation to gain additional chemical diversity. To achieve this goal, we need to find a way for efficient cellular uptake, metabolic stability and translational activity (i.e. incorporation) of useful non-canonical amino acids (ncAAs) which are usually chemically synthesized. Furthermore, we need to (re)assign coding units (i.e. codons) in the genetic code to accommodate ncAAs into target proteins.

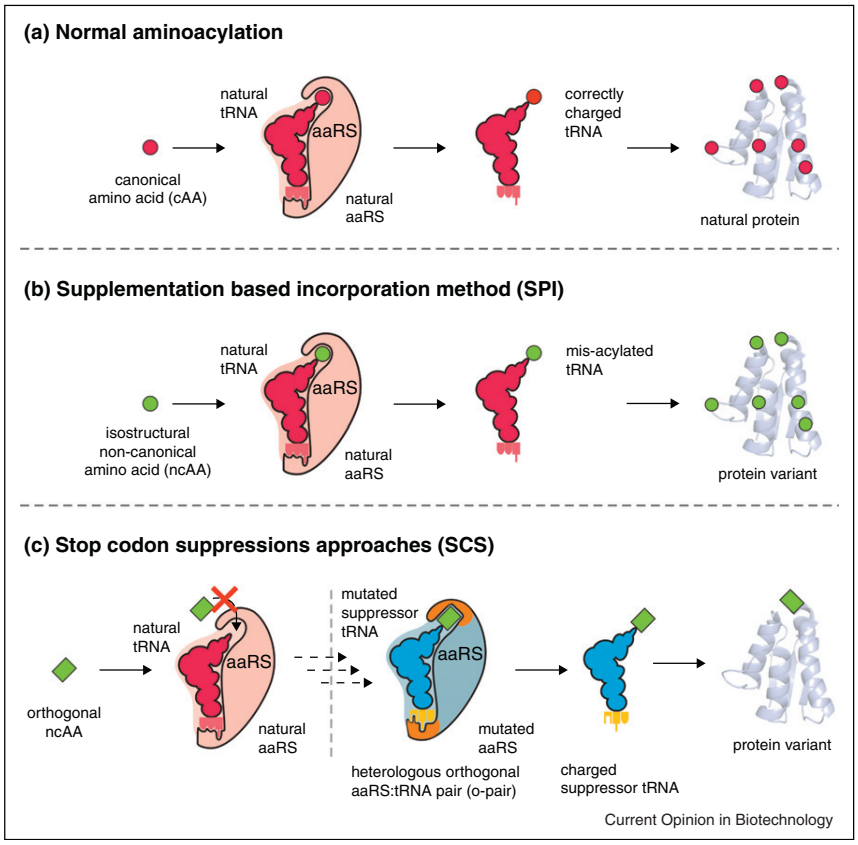
Here, we briefly sketch the most important developments in the field in the last three years. For further and more comprehensive information we refer to other recently published reviews which give an excellent overview of the methods, the incorporated ncAAs, and their diverse applications [1••,2••,3,4•,5•].

General *in vivo* incorporation strategies

ncAAs for protein engineering can be divided into two groups: first, amino acids which are isostructural to cAAs and therefore recognized by the endogenous host cell machinery, and second, amino acids which are orthogonal to the host cell system because they do not participate in conventional translation. To exploit the beneficial features of both ncAA groups for protein engineering, two distinct *in vivo* approaches are available for their co-translational incorporation (see Figure 1).

For isostructural ncAAs, residue-specific replacement of cAAs is performed with the supplementation-based incorporation method (SPI) using auxotrophic host strains [6–8]. In contrast, orthogonal ncAAs are added to the amino acid repertoire by site-specific incorporation in response to stop or quadruplet codons (stop codon suppression, SCS) using orthogonal aminoacyl-tRNA synthetase:tRNA pairs (o-pairs) [9,10]. Both approaches have particular advantages and pitfalls, depending on the specific biological questions and problems being addressed. Biological functions based on collective effects of many residues (e.g. activity [11], stability [12,13], or conformational preferences [14]) are particularly well studied and tailored by

Figure 1



Aminoacylation with canonical and non-canonical amino acids for protein translation. **(a)** In the natural scenario, tRNA aminoacylation is catalyzed by the corresponding aminoacyl-tRNA synthetase (aaRS) responsible for charging the tRNA with the cognate amino acids. **(b)** The supplementation-based incorporation method (SPI) exploits the natural substrate tolerance of the endogenous host aaRSs by using auxotrophic host strains. This allows the simultaneous exchange of many residues in a target protein by sense-codon reassignment [57]. The substrates for this procedure are non-canonical amino acids (ncAAs) which are isostructural to their canonical counterparts (i.e. atomic mutation concept [58]). **(c)** Stop codon suppression methodologies (SCS) are nominally site-specific and make use of a heterologous orthogonal aaRS:tRNA pair (o-pair) to incorporate an orthogonal amino acid in response to a stop or quadruplet codon. At this level, orthogonality is defined by a lack of cross-reactivity between the o-pair (including the ncAA) and the endogenous host synthetases, amino acids and tRNAs. Orthogonality of the o-pair is based on species-specific differences in tRNA recognition by the aaRS. The o-pair has to be evolved to enable the participation of a desired orthogonal ncAA in ribosome mediated protein synthesis (reviewed in [1••]).

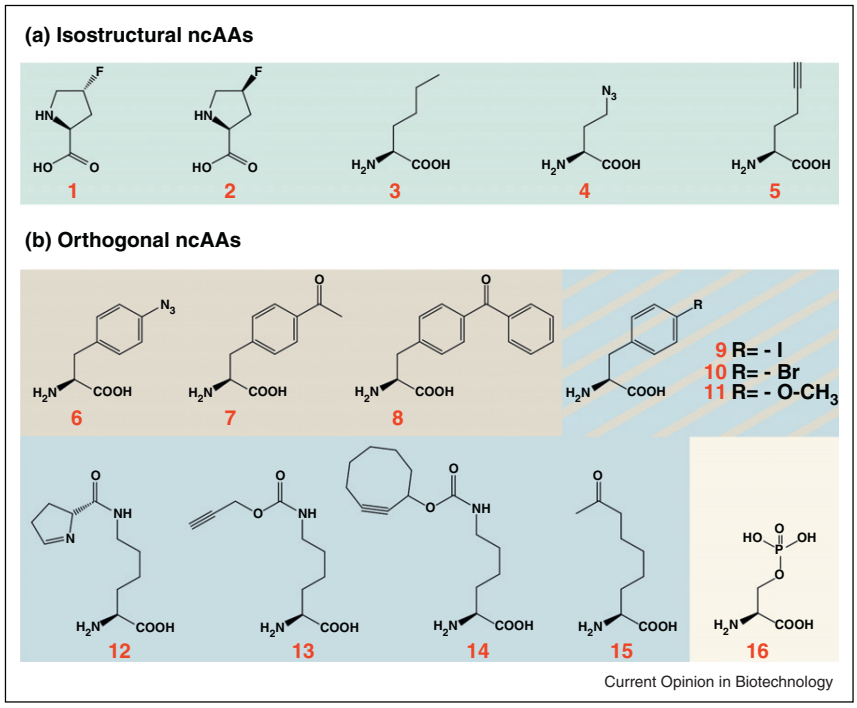
SPI. In contrast, the site-specific SCS approach enables the punctual dissection and modification of proteins (e.g. [15,16]).

Methanocaldococcus jannaschii TyrRS versus *Methanosarcinaceae* PylRSs

Since the introduction of orthogonality as a tool for protein engineering by Furter [9], various o-pairs were developed (see [1••] and [17] for comprehensive lists). Nevertheless, until recently the most frequently used o-pair was based on the TyrRS from *Methanocaldococcus jannaschii* (*mj*TyrRS). Altogether, the incorporation of around 40 different ncAAs, mainly aromatic analogs of Phe and Tyr, was reported [1••]. Among these, 4-benzoylphenylalanine (Bpa) for crosslinking [18] or 4-acetylphenylalanine (ActF) [19] and 4-azidophenylalanine (AzF) [20] for site-specific coupling reactions are certainly the most popular ones.

Now we are witnessing the spreading utilization of pyrrolysyl-tRNA synthetase (PylRS) and its cognate suppressor tRNA *pylT* from *Methanosarcinaceae* species in the whole field. In the native context, this 'natural o-pair' enables cells to incorporate the mostly aliphatic Pyl into target proteins in response to the amber (UAG) stop codon. In contrast to *mj*TyrRS, however, PylRSs already naturally display a broad substrate tolerance towards orthogonal ncAAs [21]. Within a very short time, a large number of different PylRS variants for incorporation of highly valuable aliphatic ncAAs were developed (see Figure 2 and [4•]). Remarkably, the evolved Pyl o-pair systems show a very high flexibility with respect to amino

Figure 2



Survey of some important ncAAs incorporated by SPI or SCS methods. **(a)** Isostructural ncAAs incorporated by SPI: (1) (4*s*)-fluoroproline ((4*s*)-FPro) [12], (2) (4*R*)-fluoroproline ((4*R*)-FPro) [13], (3) norleucine (Nle) [59], (4) azidohomoalanine (Aha) [60], (5) homopropargylglycine (Hpg) [61]. 1 and 2 are incorporated in response to Pro codons while 3–5 are substrates for the endogenous MetRS. **(b)** Orthogonal ncAAs incorporated by SCS: (6) 4-azidophenylalanine (AzF) [62], (7) 4-acetylphenylalanine (ActF) [63], (8) 4-benzoylphenylalanine (Bpa) [64], (9) 4-iodophenylalanine (IF) [22,65], (10) 4-bromophenylalanine (BrF) [22,66], (11) *O*-methyltyrosine (OmeY) [10,23], (12) pyrroline-carboxy-lysine (Pcl) [56••], (13) *N6*-[(2-propynyloxy)-carbonyl]-lysine (PoxK) [67], (14) *N6*-[(cyclooct-2-yn-1-yloxy)carbonyl]-lysine (CoK) [68], (15) 2-amino-8-oxononanoic acid (KetoK) [69], (16) *O*-phosphoserine (Sep) [54••]. 6–8 are incorporated by variants of *mj*TyrRS, 12-15 by variants of PylRS, and 9–11 were incorporated by both systems. Currently available PylRS-based o-pairs are derived from *Methanosarcina barkeri* [70], *Methanosarcina mazei* [71], and *Desulfitobacterium hafniense* [72]. *O*-phosphoserine was genetically encoded by a *Methanococcus maripaludis* SepRS:*mj*tRNA$^{Cys}_{CUA}$ o-pair and a mutated EF-Tu. If available, particular applications were cited for the presented ncAAs. For a more comprehensive view we refer to reviews [1••,2••,3,4•,5•].

acid charging. For example, the groups of Wang and Liu evolved PylRSs for aromatic Phe and Tyr analogs (see Figure 2 and [22,23]). Most importantly, the system is very tolerant in regard to different tRNA anticodons. UAG, UAA, UGA, UAGA were read without further adaptations in the tRNA^{Pyl} [24]. Doubtlessly, the PylRS systems are not only highly valuable tools for ncAA incorporation but are also superior to *mj*TyrRS systems. However, it should always be kept in mind that selection of orthogonal aaRS variants for new ncAAs is still a laborious and time consuming task.

Another important issue of o-pairs tackled only recently is the degree of substrate tolerance in these systems. Like endogenous aaRSs, orthogonal aaRSs can also display significant substrate tolerance. In contrast to SPI, the whole system maintains orthogonality towards all cAAs. Remarkably, Young *et al.* found that *mj*TyrRS evolved for 4-cyanophenylalanine can incorporate 18 different Phe and Tyr analogs [25]. Similarly, Miyake-Stoner *et al.* generated *mj*TyrRSs capable of incorporating a variety of differently fluorinated 4-methylphenylalanines which are useful in ^{19}F NMR studies [26]. These studies nicely demonstrate that the re-screening of previously available o-pairs can also be a strategy to identify an aaRS capable of charging a desired ncAA.

Parallel incorporation of multiple ncAAs in a single expression experiment

The incorporation of a single ncAA at a single position in a protein is certainly useful as tool to study particular biological problems (e.g. receptor–ligand interactions [27]). However, it is often not sufficient for many academic and biotechnological applications. This will leverage the whole field towards the introduction of two or more reactive handles or probes into recombinant proteins. The first experiment was reported by Schultz and coworkers [28] followed by recent works of Chin and Liu (reviewed in [29•]). The combination of *mj*TyrRS and *Methanosarcina mazei* PylRS derived o-pairs enabled the simultaneous reassignment of an amber stop and an ochre (UAA) [24] or quadruplet [30] codon. However, the

reassignment of further codons using SCS methodologies is currently impeded by two crucial limitations. First, suppressor tRNAs have to compete with the endogenous release factors RF1 and RF2 during translation at the ribosome. Second, frame-shift suppression is still very inefficient [31].

In contrast, a multiple incorporation of three and even more ncAAs into a single target protein is possible by SPI without substantial loss of protein yield. For example, the parallel incorporation of homopropargylglycine (Hpg, click handle), 4-azatryptophan (fluorescence tag), and (4*s*)-fluoroproline ((4*s*)-FPro) (stabilizing amino acid) yielded active tailor-made protein [32•]. Similarly, a highly fluorinated active lipase with amino acid exchanges at as many as 24 positions was reported [33]. We also explored the potential of multi-labeling by SPI in combination with site-specific SCS. In particular, Bpa along with norleucine or (4*s*)-FPro incorporation provided a useful combination of desired features in the recombinant protein [34]. At the same time, Yun and coworkers expressed a green fluorescent protein (GFP) variant harboring 3,4-dihydroxy-phenylalanine and Hpg or azidohomoalanine (Aha) using the same strategy [35].

Engineering of bacterial strains free of release factors or particular termination codons

The major challenge in the further development of all suppression-based methodologies is to achieve unlimited reassignments of the codons of interest, for example the amber stop codon. The main obstacles include release factor competition, catalytic performance of o-pairs and poorly understood mRNA context effects. In this regard, systems based on amber suppression have been considerably improved in the last years. These improvements include first, the use of more suppressor tRNA copies [36], second, optimized suppressor tRNAs for enhanced ncAA-tRNA binding to elongation factor Tu (EF-Tu) [37], third, enhanced co-expression plasmids for higher aaRS expression [38,39], and finally, the co-expression of the C-terminal domain of the ribosomal protein L11 [40]. Furthermore, the decreased affinity of orthogonal ribosomes towards RF1 led to higher amber suppression [41]. Despite these important improvements, the expression of target proteins with more than three in-frame amber stop codons had yet to be reported.

Doubtlessly, the most appealing idea to efficiently eliminate this drawback for the amber stop codon was the removal of RF1 from *Escherichia coli* since it would eliminate the competition reaction with the amber suppressor tRNA during translation. However, the *prfA* gene coding for RF1 is essential in *E. coli* [42]. Therefore, Church and coworkers set out to exchange all 314 TAG codons in the *E. coli* genome by TAA and remove RF1, subsequently [43]. In their study, they succeeded in generating four strains with around 80 complementary TAG to TAA mutations each. Unfortunately, the final step to unify these four subsets to one TAG free *E. coli* strain and the RF1 knockout was not performed in the study.

In contrast to this genome remodeling approach, the teams of Yokoyama/Sakamoto [44•] and Lei Wang [45•] came up with two different genetic strategies to solve the 'RF1 problem' without the removal of all TAG codons from the genome. In the frame of the first strategy it was speculated that the degradation of essential proteins due to ribosome stalling at unassigned UAG codons [46] may be a possible reason for RF1 essentiality. Thus, the *E. coli* strain was *trans* complemented with TAA ending versions of seven essential genes originally terminated with TAG. In the second step, an engineered o-pair reading UAG was introduced. Host cells configured in this way, allowed successful knockout of *prfA*. Not surprisingly, an enhanced version of this Δ*prfA* strain [47] enabled glutathione S-transferase expression with different ncAAs at up to seven amber positions in parallel. In contrast, the second strategy assumes that insufficient termination at UAA instead of UAG codons (RF1 and RF2 are both reading UAA) upon *prfA* knockout causes RF1 essentiality. Indeed, by enhancing RF2 expression and activity, Wang and coworkers could successfully knockout *prfA* [45•] from a genome reduced *E. coli* strain [48]. The approach enabled the successful read-though of 10 UAG codons in GFP.

It is still difficult to rationalize how these two different strategies can be reconciled. However, the Δ*prfA* strain designed by Wang and coworkers has the advantage of being independent of *trans* complementation. This permits the facile use of already available expression systems with o-pairs virtually without limitations.

New orthogonal pairs and mutually orthogonal *mj*TyrRSs

The successful knockout of RF1 leads to the liberation of the amber codon for complete reassignment. However, multiple *in vivo* incorporations with distinct ncAAs will need a further extended number of blank codons. A quite popular opinion in the academic community is that this 'lack of blank codons' could be solved by introducing quadruplet codons [49]. In addition, we are convinced that the liberation of some (rare) codons from their natural assignments (i.e. codon emancipation) might also be very powerful to obtain new blank codons for reassignment to ncAAs [50]. In any case, many different (and mutually orthogonal) o-pairs from various sources will be needed for this task. Therefore, a lot of effort should be made in the further development of existing o-pairs and the search for natural ones. For example, Hughes *et al.* rationally designed an o-pair based on *Saccharomyces cerevisiae* TrpRS:$tRNA^{Trp}$ for use in *E. coli* [17]. Furthermore, the *Caulobacter crescentus* HisRS:$tRNA^{His}$ pair was identified as a natural o-pair in *E. coli* [51]. Finally, starting from

the parent *mj*TyrRS:tRNA$^{Tyr}_{CUA}$ o-pair, two mutually orthogonal systems were developed recently [52•].

It should be kept in mind that only the design of o-pairs and their use in suitable host cells will not be sufficient for incorporation of many desired ncAAs. For example, the issue of ncAA-tRNA interaction with EF-Tu was not considered for a long time in the whole field. Sisido and co-workers were the first ones to discover that it is possible to improve EF-Tu interaction with various ncAA-tRNAs *in vitro* [53]. The most striking example for EF-Tu interaction engineering was recently provided by Söll and coworkers [54••]. In particular, they developed an amber suppression based co-translational *O*-phosphoserine (Sep) incorporation system starting from a natural pathway in *Methanococcus maripaludis*. The system is based on a naturally orthogonal SepRS and its establishment included first, engineering of a cognate amber suppressor tRNA^{Sep}, second, screening for EF-Tu mutants capable of binding Sep-tRNA^{Sep}, and finally, host cell engineering to block endogenous phosphoserine phosphatase activity. Interestingly, the strategy for designing the Sep encoding system was borrowed from a natural example, since natural selenocysteine incorporation includes a specialized elongation factor as well (reviewed in [55]).

Outlook — codon emancipated cells with maximum chemical liberty

The rapid development of new orthogonal pairs and new aaRS specificities for various ncAAs discussed in this review will allow the assignment of novel functionalities of the genetic code with a maximum degree of chemical liberty. Orthogonal pairs should be designed to serve as generalist tools so that ncAAs-mediated protein engineering will not only be relevant for single recombinant proteins, but also feasible throughout the entire *E. coli* proteome. The ground-breaking works of Dieter Söll on GluRS systems clearly highlight how codons can be emancipated and liberated from the current function [50]. In addition, the use of genome remodeling [43] will enable stable and valuable ncAA additions to the entire proteome of a cell. As the whole research area moves towards maturity, more and more approaches will contribute to solve industrially relevant bio-production problems, including advanced peptide and protein production.

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- •• of outstanding interest

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