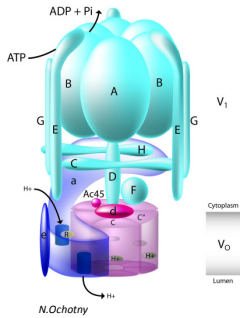
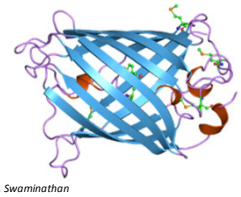
Synthetic life SL5

CHAPTER 2

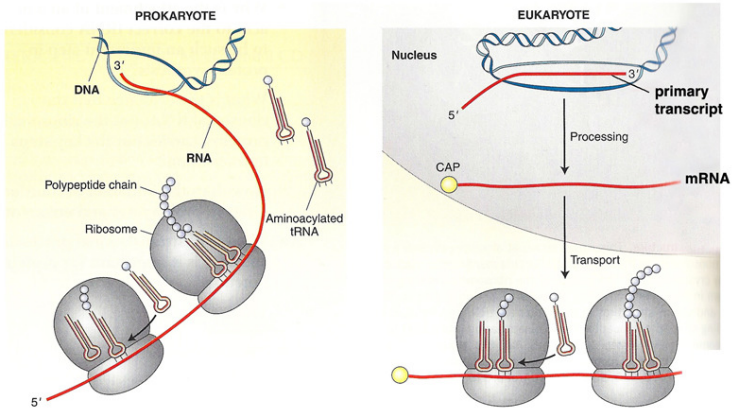




PROTEINS

Part 1 – genetic encoding of non-standard aminoacids

Translation: RNA → proteins

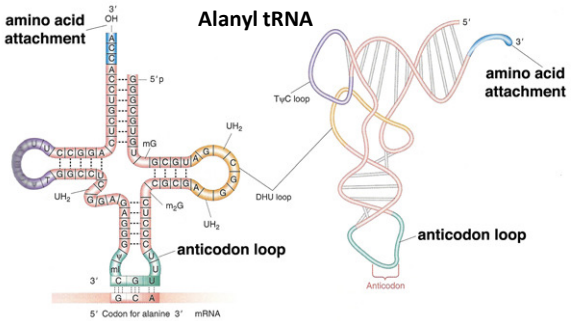


Translation: RNA → proteins

A transfer RNA has a cloverleaf structure with regions of base pairing.
A tRNA has the structure shown here both as a flat cloverleaf and in its folded form.

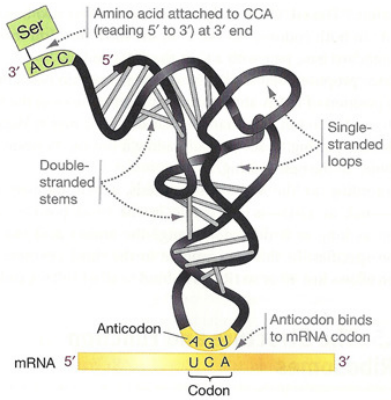
Two important parts of a tRNA:

- the anticodon, which participates in base pairing with a codon in the mRNA
- the site of amino acid attachment at the 3' end of the tRNA



Translation: RNA → proteins

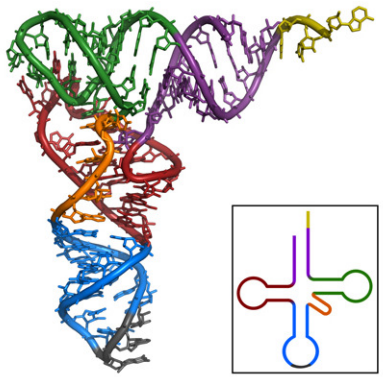
Charged serine tRNA



This shows a "charged" serine tRNA, covalently attached to the amino acid serine at its 3' end, with the anticodon paired to a serine codon

Translation: RNA → proteins

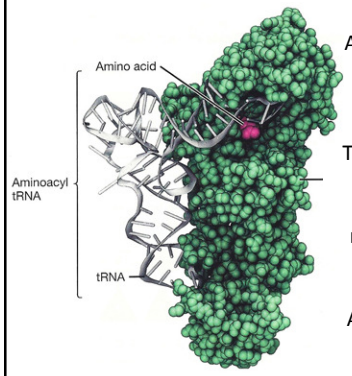
tRNA in 3D



This is a better representation of the 3D structure of a tRNA. The model is color-coded to the flat cloverleaf representation in the lower right

Translation: RNA → proteins

Aminoacyl tRNA synthethase



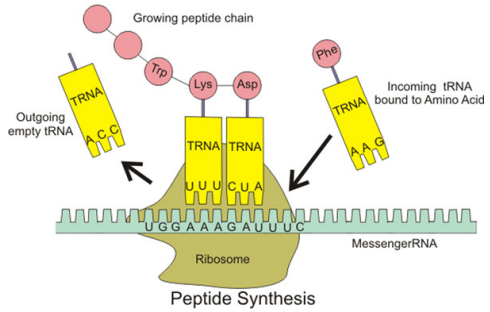
A special set of enzymes "charges" tRNAs, attaching the correct amino acid to particular tRNAs.

A charged tRNA is called an aminoacyl tRNA, so the charging enzymes are more properly called aminoacyl tRNA synthetases.

There is only one aminoacyl tRNA synthetase for each amino acid, even though there can be multiple tRNAs for that amino acid. Each aminoacyl tRNA synthetase is able to recognize all of the tRNAs that need to be charged with the one amino acid that is their specialty.

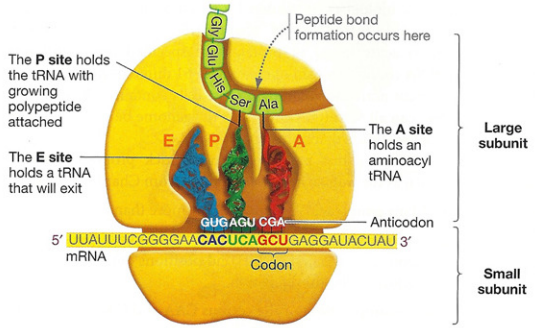
Amino acids are attached to the hydroxyl (-OH) group at the 3' end of the tRNA through their carboxyl (-COOH) group

Translation: RNA → proteins



Proteins are synthesized by ribosomes that read the sequence of mRNA and write it as protein. Translation is accomplished with the help of charged tRNAs that allow individual codons to specify the next amino acid added to the growing polypeptide. The mRNA is read from the 5' end to the 3' end, with the protein being synthesized from the amino terminus to the carboxy terminus

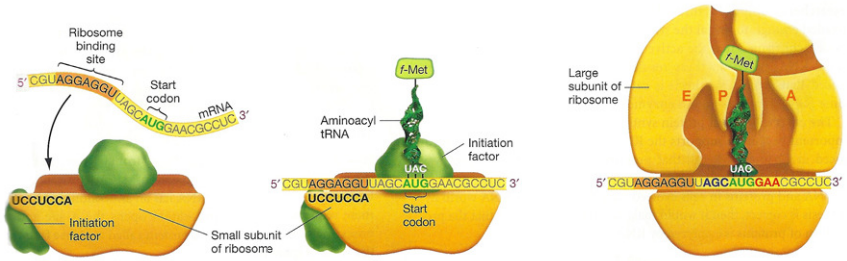
Translation: RNA → proteins



Three sites are associated with tRNAs: the A (aminocyl) site, that accepts a new aminoacyl tRNA; the P (polypeptide) site, that holds a tRNA with the growing polypeptide chain; and the E (exit) site that holds an uncharged tRNA ready to exit the ribosome

Translation: RNA → proteins

Translation initiation



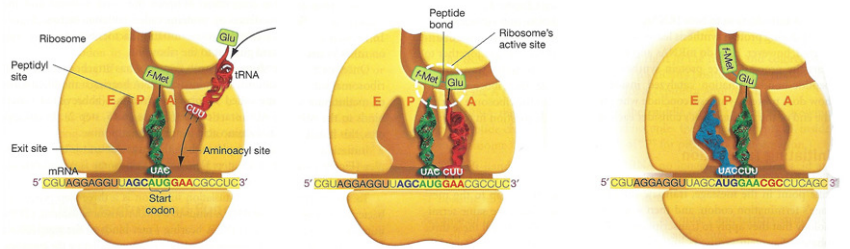
1. mRNA binds to small subunit. Ribosome binding site sequence binds to a complementary sequence in an RNA molecule in the small subunit of the ribosome, with the help of protein initiation factors.

2. Initiator aminoacyl tRNA binds to start codon.

3. Large subunit of ribosome binds, completing ribosome assembly. Translation begins.

Translation: RNA → proteins

Translation elongation



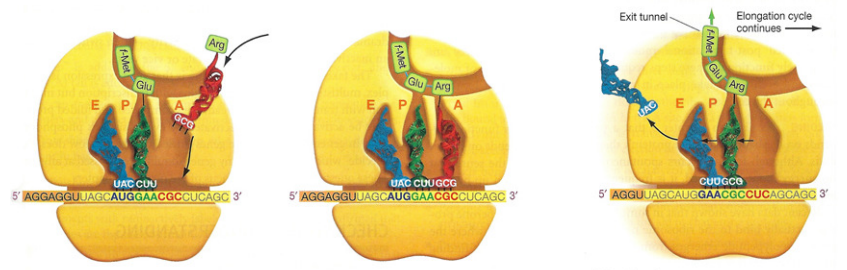
1. Incoming aminoacyl tRNA New tRNA moves into A site, where its anticodon base pairs with the mRNA codon.

2. Peptide bond formation The amino acid attached to the tRNA in the P site is transferred to the tRNA in the A site.

3. Translocation mRNA is ratcheted through the ribosome by elongation factors (not shown). The tRNA attached to the polypeptide chain moves into the P site. The A site is empty.

Translation: RNA → proteins

Translation elongation



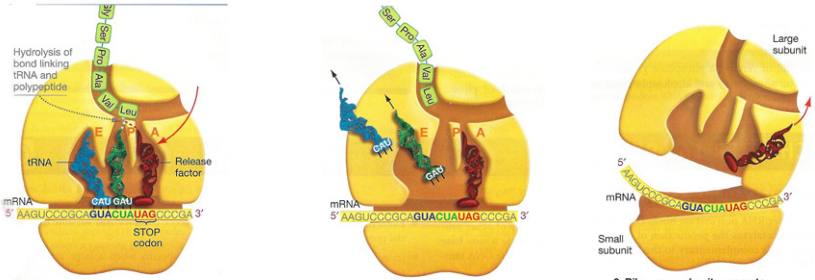
4. Incoming aminoacyl tRNA New tRNA moves into A site, where its anticodon base pairs with the mRNA codon.

5. Peptide bond formation The polypeptide chain attached to the tRNA in the P site is transferred to the aminoacyl tRNA in the A site.

6. Translocation mRNA is ratcheted through the ribosome again. The tRNA attached to polypeptide chain moves into P site. Empty tRNA from P site moves to E site, where tRNA is ejected. The A site is empty again.

Translation: RNA → proteins

Translation termination



1. Release factor binds to stop codon. When translocation exposes a stop codon, a release factor fills the A site. The release factor breaks the bond linking the tRNA in the P site to the polypeptide chain.

2. Polypeptide is released. The hydrolysis reaction frees the polypeptide, which is released from the ribosome. The empty tRNAs are released either along with the polypeptide or...

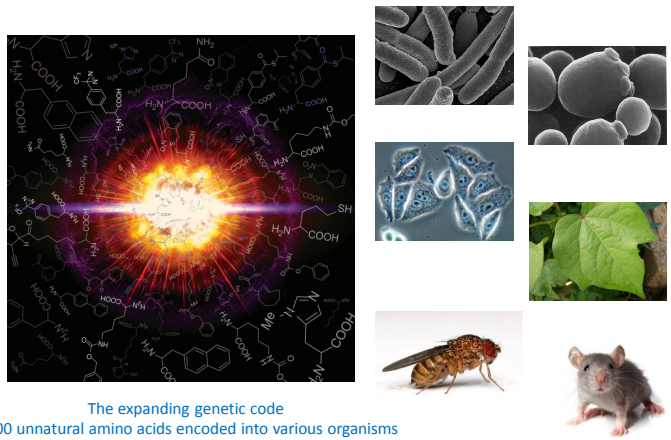
3. Ribosome subunits separate. ...when the ribosome separates from the mRNA, and the two ribosomal subunits dissociate. The subunits are ready to attach to the start codon of another message and start translation anew.

Translation: RNA → proteins – the genetic code

nonpolar polar basic acidic (stop codon)

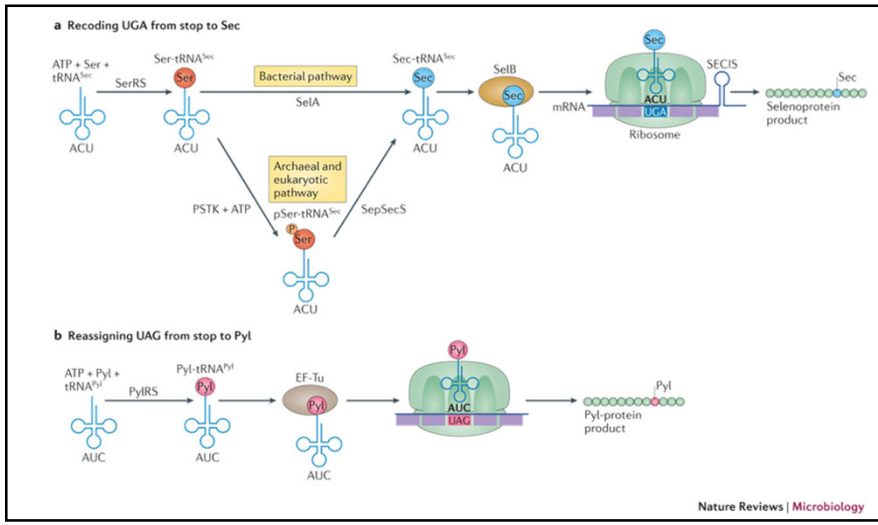
Standard genetic code

1st base	2nd base U		2nd base C		2nd base A		2nd base G		3rd base
U	UUU	(Phe/F) Phenylalanine	UCU	(Ser/S) Serine	UAU	(Tyr/Y) Tyrosine	UGU	(Cys/C) Cysteine	U
	UUC		UCC		UAC		UGC		C
	UUA	(Leu/L) Leucine	UCA		UAA	Stop (*Ochre*)	UGA	Stop (*Opal*)	A
	UUG		UCG		UAG	Stop (*Amber*)	UGG	(Trp/W) Tryptophan	G
C	CUU		CCU	(Pro/P) Proline	CAU	(His/H) Histidine	CGU	(Arg/R) Arginine	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	(Gln/Q) Glutamine	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	(Ile/I) Isoleucine	ACU	(Thr/T) Threonine	AAU	(Asn/N) Asparagine	AGU	(Ser/S) Serine	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	(Lys/K) Lysine	AGA	(Arg/R) Arginine	A
	AUG[A]	(Met/M) Methionine	ACG		AAG		AGG		G
G	GUU	(Val/V) Valine	GCU	(Ala/A) Alanine	GAU	(Asp/D) Aspartic acid	GGU	(Gly/G) Glycine	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	(Glu/E) Glutamic acid	GGA		A
	GUG		GCG		GAG		GGG		G

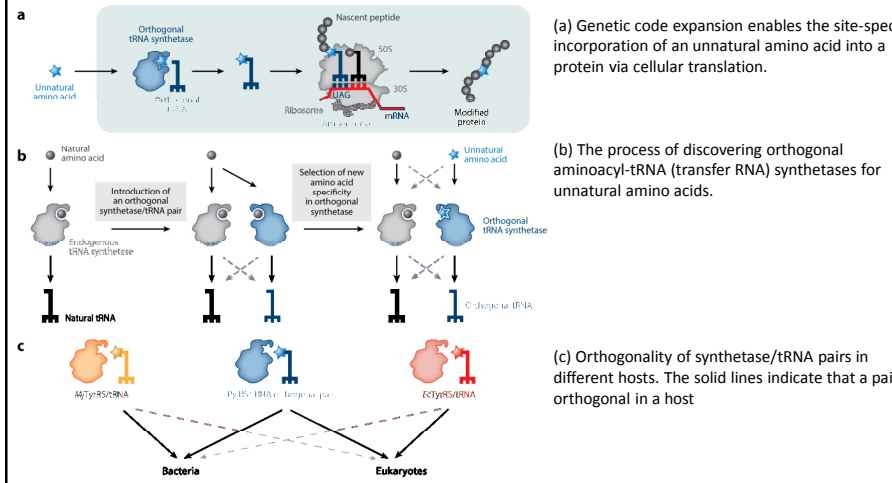


The expanding genetic code
> 200 unnatural amino acids encoded into various organisms

Ura Y, Beierle J, Leman L, Orgel LE, Ghadiri MR. *Science* **2009**, *325*, 73-77.



The expanding genetic code

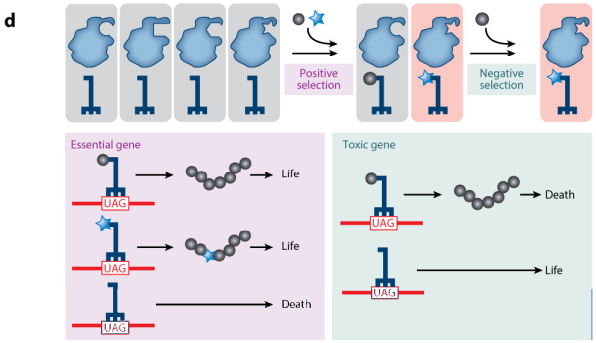


(a) Genetic code expansion enables the site-specific incorporation of an unnatural amino acid into a protein via cellular translation.

(b) The process of discovering orthogonal aminoacyl-tRNA (transfer RNA) synthetases for unnatural amino acids.

(c) Orthogonality of synthetase/tRNA pairs in different hosts. The solid lines indicate that a pair is orthogonal in a host

(*d*) Sequential positive and negative selections enable the discovery of synthetase/tRNA pairs that direct the incorporation of unnatural amino acids.

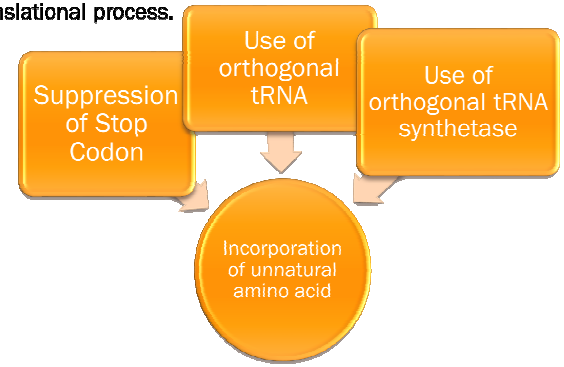


Chin JW. 2014.
Annu. Rev. Biochem. 83:379–408

***Ec*TyrRS,** *Escherichia coli* tyrosyl-tRNA synthetase;
***Mj*TyrRS,** *Methanococcus janaschii* tyrosyl-tRNA synthetase;
mRNA, messenger RNA; **PylRS,** pyrrolysyl-tRNA synthetase.

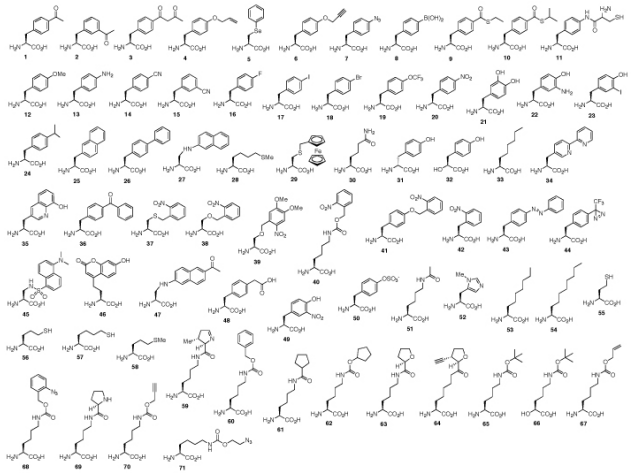
The expanding genetic code

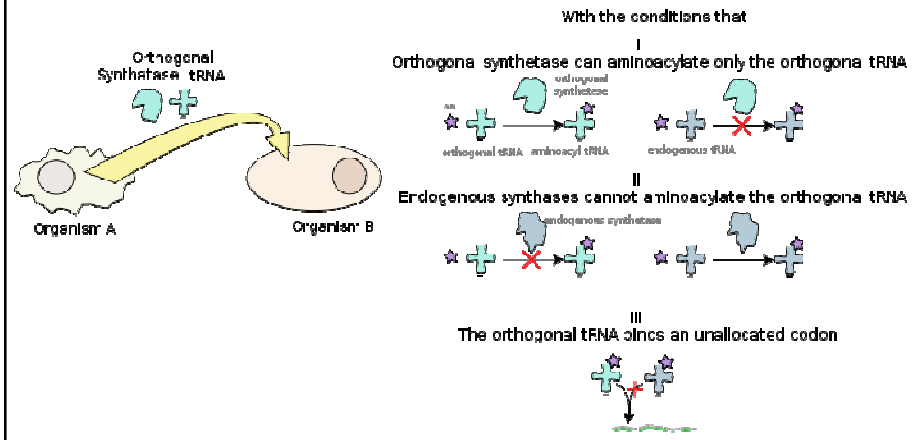
Incorporation of unnatural amino acid into protein is a pre-translational process.

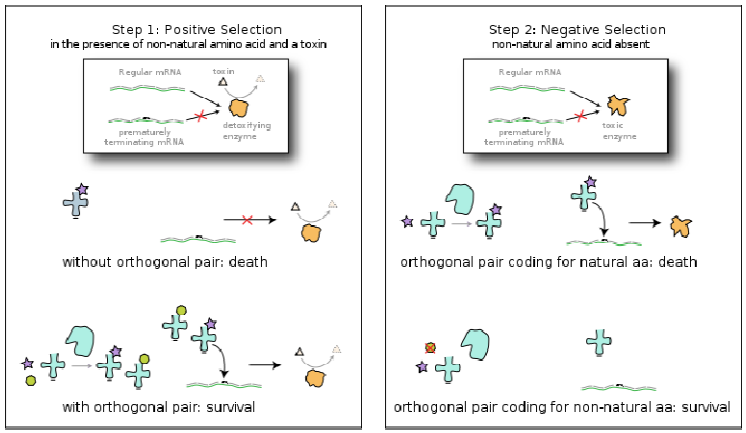


Bacteria do not have post-translational modifications. They lack the enzymes that cleave peptide, attach carbohydrates, and make chemical modifications.

The expanding genetic code

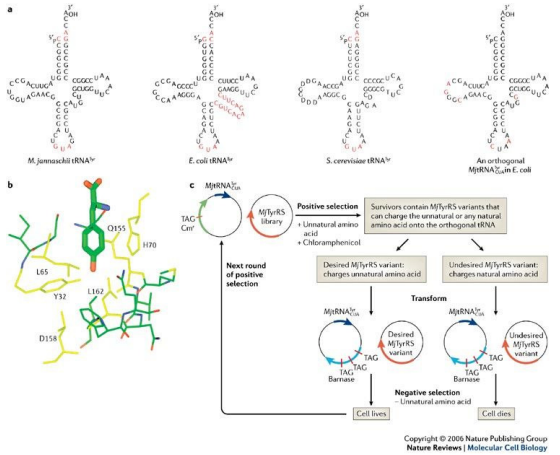






Ura Y, Beierle J, Leman L, Orgel LE, Ghadiri MR. *Science* **2009**, *325*, 73-77.

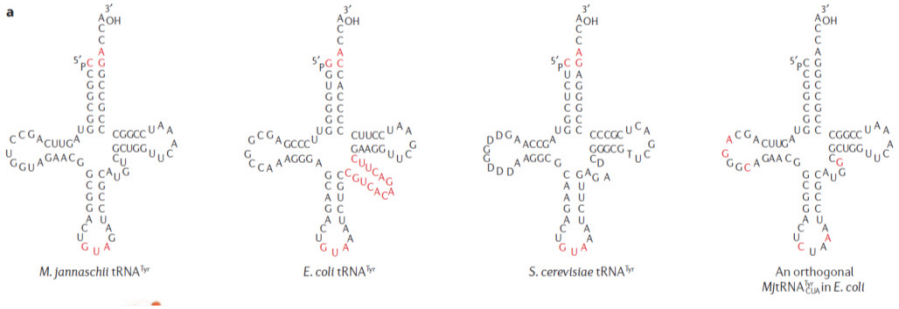
The expanding genetic code



J. Xie, P. G. Schultz *Nature Rev. Mol. Cell Biol.* **2006**, *7*, 775-782.

The expanding genetic code

The development of an orthogonal amber suppressor *Methanococcus jannaschii* tyrosyl–transfer-RNA (*Mj* $tRNA^{Tyr}_{CUA}$) in *Escherichia coli* and the modification of the amino-acid specificity of its cognate *M. jannaschii* tyrosyl–tRNA synthetase (*Mj*TyrRS)



The tRNA^{Tyr} molecules from *M. jannaschii*, *E. coli* and *Saccharomyces cerevisiae* (with the key identity elements that are recognized by the cognate synthetases highlighted in red), and the orthogonal amber suppressor *Mj* $tRNA^{Tyr}_{CUA}$ in *E. coli* (with the modified nucleotides highlighted in red). The D nucleotide is dihydrouridine.

J. Xie, P. G. Schultz *Nature Rev. Mol. Cell Biol.* **2006**, *7*, 775-782.

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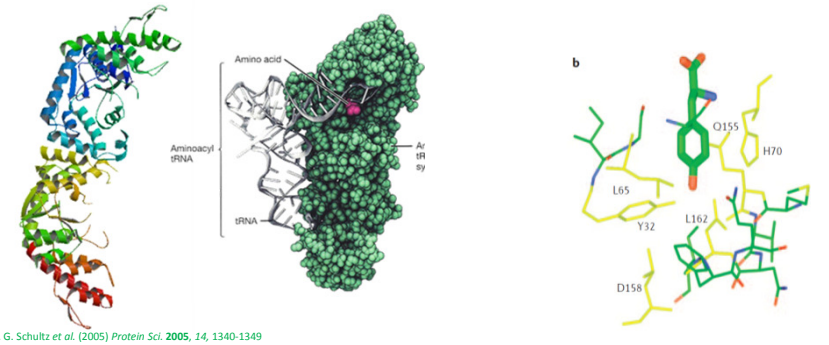


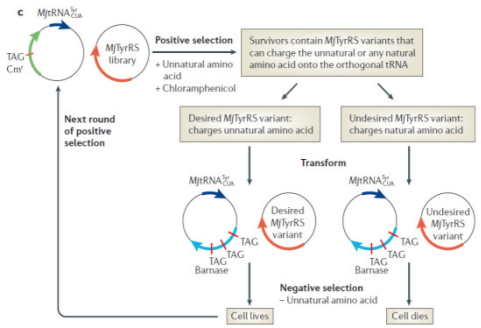
P. G. Schultz *et al.* (2005) *Protein Sci.* **2005**, *14*, 1340-1349

A library of MjTyrRS mutants was generated by randomly mutating 6 residues (shown in yellow) in the Tyr-binding site to all 20 amino acids. Tyr is shown in its binding site using a thicker stick representation.

J. Xie, P. G. Schultz *Nature Rev. Mol. Cell Biol.* **2006**, *7*, 775-782.

The expanding genetic code

A general positive and negative selection scheme for the development of synthetase variants that are specific for an unnatural amino acid in E. coli.

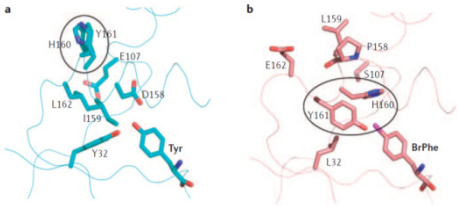


Following the generation of a large library (~10^9 mutants) of, in this case, MjTyrRS active-site mutants, positive and negative selections were carried out. The positive selection was based on resistance to chloramphenicol, which was conferred in the presence of MjTyrRS and the unnatural amino acid (or any natural amino acid that the MjTyrRS could charge onto the orthogonal tRNA) by the suppression of an amber mutation (TAG) at a permissive site in the chloramphenicol acetyltransferase gene (labelled Cm^r). The negative selection used the toxic barnase gene with amber mutations at permissive sites and was carried out in the absence of the unnatural amino acid. Only MjTyrRS variants that could acylate the orthogonal $tRNA^{Tyr}_{CUA}$ with the unnatural amino acid and not with the endogenous amino acids could survive both selections.

J. Xie, P. G. Schultz *Nature Rev. Mol. Cell Biol.* **2006**, *7*, 775-782.

The expanding genetic code

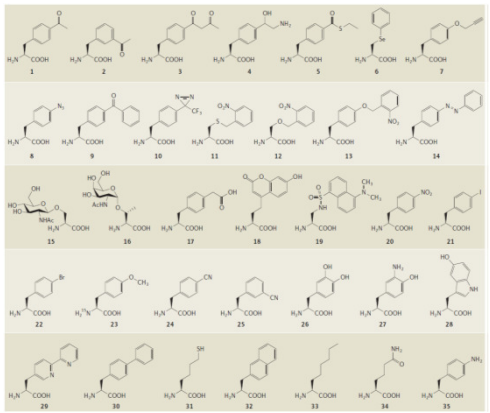
The structures of the wild-type and a mutant Methanococcus jannaschii tyrosyl–tRNA synthetase bound to their cognate amino acids.



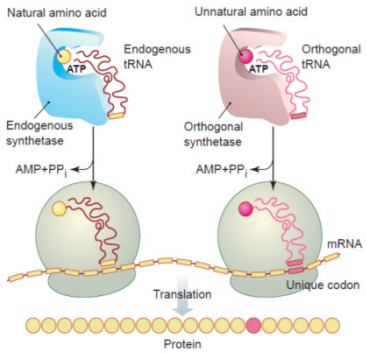
a The active site of wild-type *Methanococcus jannaschii* tyrosyl–transfer-RNA synthetase (MjTyrRS) bound to Tyr.
b The active site of a mutant MjTyrRS that binds to p-bromophenylalanine (labelled BrPhe in the figure). The active site of the mutant contains the mutations Y32L, E107S, D158P, I159L and L162E. The active-site D158P and Y32L mutations remove two hydrogen bonds to the hydroxyl group of the Tyr side chain, which disfavours the binding of the natural substrate. The D158P mutation results in the termination of helix α8 and produces significant translational and rotational movements of several active-site residues. These effects, in conjunction with the effects of the Y32L mutation, lead to an expanded hydrophobic active-site cavity that favours the binding of p-bromophenylalanine. Black frames highlight the different positioning of H160 and Y161 in these structures.

J. Xie, P. G. Schultz *Nature Rev. Mol. Cell Biol.* **2006**, *7*, 775-782.

The expanding genetic code



J. Xie, P. G. Schultz *Nature Rev. Mol. Cell Biol.* **2006**, *7*, 775-782.



New building blocks. A general method for genetically encoding unnatural amino acids into proteins.

L. Wang *Science* **2003**, *302*, 584-585.

1. METHANOCCOCUS JANNASCHII (MJ)

- Tyrosyl tRNA and synthetase
- Similar Recognition Elements as E. coli
- No cross reaction with E. coli machinery

2. WHY USE TAG (UAG CODON)

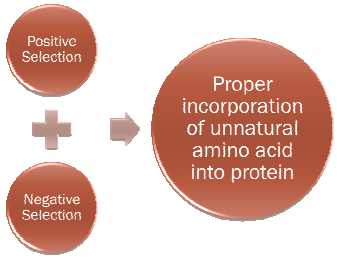
Essentially, TAG was used for amber suppression because of three very important points:

1) The tRNAs can sufficiently translate amber suppression of this codon,
2) TAG is a rarely used or found stop codon found in bacteria and yeast, so it rarely terminates genes, and
3) The lack of termination of the gene will not alter the growth of the organism.

3. Modification of synthetase to accommodate unnatural amino acid

Direct evolution method was implemented in order to rearrange the active site to accomodate the unnatural amino acid.

1. A library of 10^9 possible synthetase active sites were randomized for one example.
2. Result: Active site specific to unnatural amino acid.



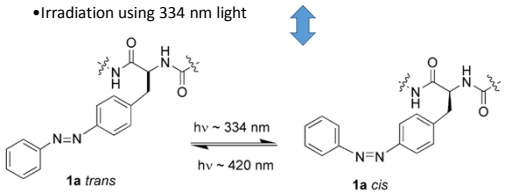
By using a library of 10^9 synthetases with alterations to their active site, positive and negative selections were performed in order to implement the unnatural amino acid into the specified protein. The positive selection requires a plasmid with the chloramphenical acetyl transferase gene composing of a TAG permissive site. This would be grown in the presence of chloramphenicol and unnatural amino acid on a dish with proper medium. The survivors would then placed in another cell to be grown in the presence of a toxic barnase gene with three permissive sites.

Incorporation of Photo-isomerizable Unnatural AA Phenylalanine-4-azobenzene

One of the first examples used by Schultz was the incorporation of a photo-isomerizable unnatural amino acid such as an Azobenzene. It is a trans-cis isomer that irradiates at 334 nm to become the least stable cis isomer, then can be irradiated back using 420 nm light to its more stable trans isomer as shown. The orthogonal pair used here will be a tyrosyl tRNA and synthetase abbreviated above.

- PHENYLALANINE-4-AZOBENZENE (AzoPhe)
- Trans
  - Most Stable
  - Irradiation using 420 nm light
- Cis
  - Least Stable
  - Irradiation using 334 nm light

- Orthogonal Pair
- Tyrosyl mutant amber suppressor tRNA
  - ($MjtRNA^{Tyr}_{CUA}$)
- Tyrosyl tRNA synthetase
  - (MjTyrRS)



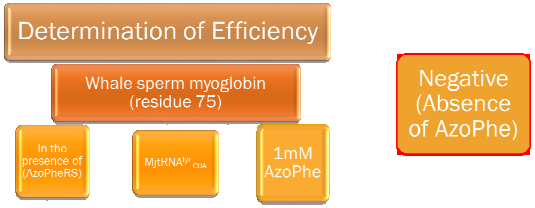
1a *trans* **1a** *cis*

Bose, M.; Groff, D.; Xie, J.; Eric, B.; Schultz, P. G. *J. Am. Chem. Soc.* **2005**, *128*, 388.

THE ACTIVE SITE OF AZOPHE SYNTHETASE

Direct evolution and positive and negative selections were made.

- Tyr-32, Leu-65, Phe-108, Gln-109, Asp-158, and Leu-162
- Tyr32Gly, Leu65Glu, Phe108Ala, Gln109Glu, Asp158Gly, and Leu162His.



In order to incorporate the unnatural amino acid into the protein, first the active site of the synthetase must be altered to accomodate the unnatural amino acid. Using the direct evolution method mentioned before, known amino acids around the active site of the synthetase were rearranged and substituted with other amino acids to result in the best accomodation for the unnatural amino acid as shown above in red. To determine the efficiency of the incorporation, whale sperm myoglobin was used in the presence of the tRNA, Synthetase, and unnatural amino acid.

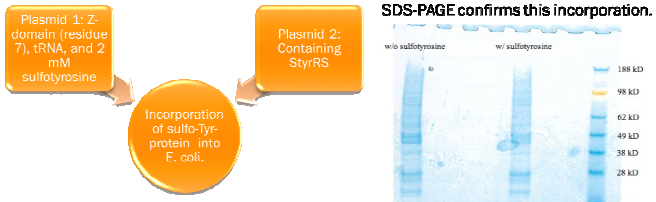
EXAMPLE 2: SULFOTYROSINE (TYS) (1) BUILDING SPECIFICITY OF SYNTHETASE AND (2) INCORPORATION INTO PROTEIN

Direct evolution and positive and negative selections were made.

Tyr32 Leu65 Asp158 Ile159 Leu162

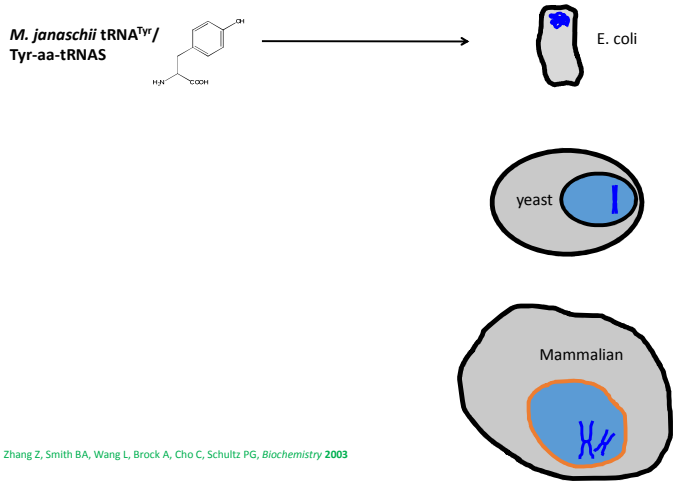
Tyr32Leu, Leu65Pro, Asp158Gly, Ile159Cys, and Leu162Lys

SDS-PAGE confirms this incorporation.

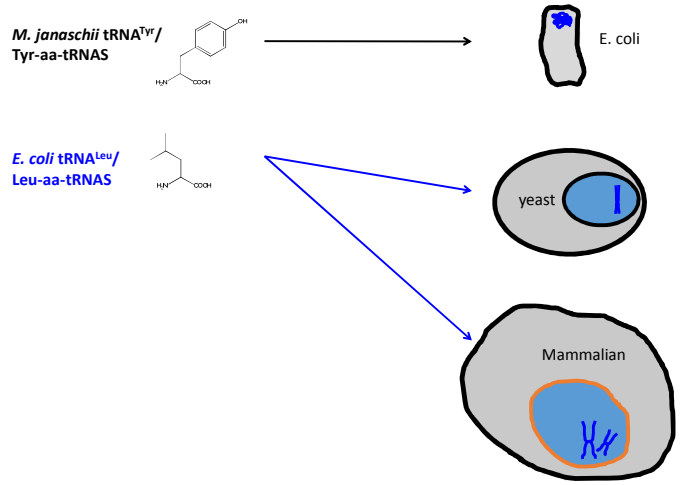


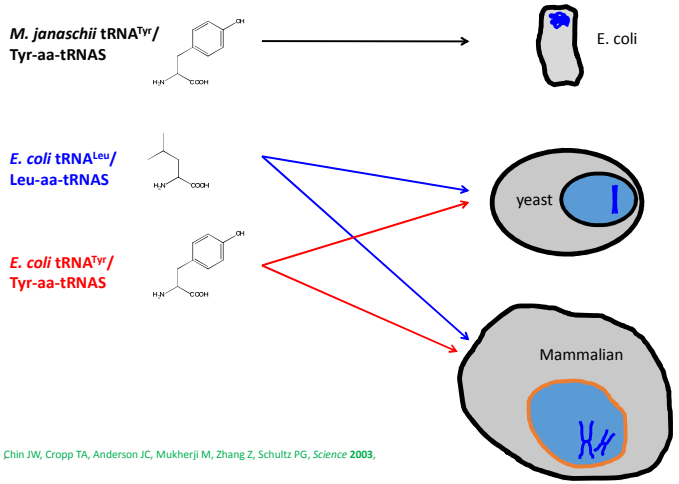
Result: Peak of 7,876 Da only sulfotyrosine.

Kehoe, J. W.; Bartoszd, C. R. Chem. Biol. 2000, 7, R57.



Zhang Z, Smith BA, Wang L, Brock A, Cho C, Schultz PG, *Biochemistry* **2003**





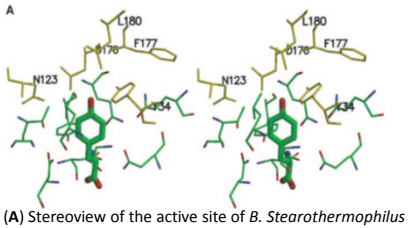
Chin JW, Cropp TA, Anderson JC, Mukherji M, Zhang Z, Schultz PG, *Science* **2003**,

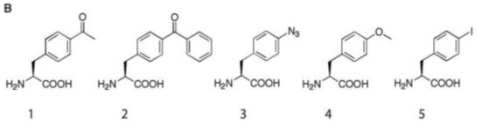
The expanded eucaryotic genetic code

E. coli tyrosyl–tRNA synthetase (TyrRS) efficiently aminoacylates *E. coli* $tRNA_{CUA}$ when both are genetically encoded in *S. cerevisiae* but does not aminoacylate *S. cerevisiae* cytoplasmic tRNAs

In addition, *E. coli* tyrosyl $tRNA_{CUA}$ is a poor substrate for *S. cerevisiae* aminoacyl–tRNA synthetases but is processed and exported from the nucleus to the cytoplasm and functions efficiently in protein translation in *S. cerevisiae*

On the basis of the crystal structure of the homologous TyrRS from *Bacillus stearothermophilus* , five residues (Fig. 1A) in the active site of *E. coli* TyrRS were randomly mutated.





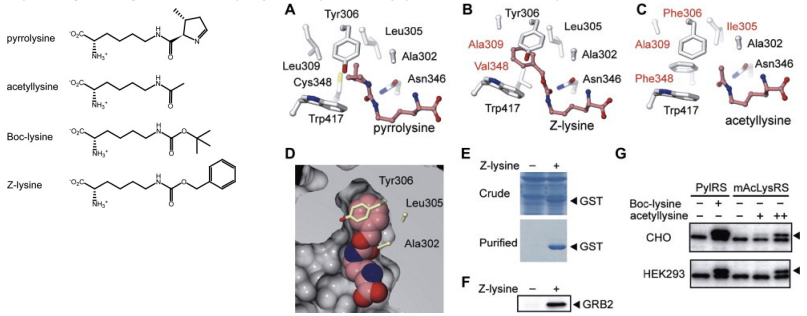
(**A**) Stereoview of the active site of *B. Stearothermophilus* tyrosyl–tRNA synthetase with bound tyrosine. The mutated residues (*E. Coli):* Tyr_{37} (*B. stearothermophilus* TyrRS residue Tyr_{34}), Asn_{126} (Asn_{123}), Asp_{182} (Asp_{176}), Phe_{183} (Phe_{177}), and Leu_{186} (Leu_{180}).

(**B**) Chemical structures of *p*acetyl-L-phenylalanine, **1**; *p*-benzoyl-L-phenylalanine, **2**; *p*-azido-L-phenylalanine, **3**; methyl-L-tyrosine, **4**; and *p*-iodo-L-tyrosine, **5**.

Chin JW, Cropp TA, Anderson JC, Mukherji M, Zhang Z, Schultz PG, *Science* **2003**, *301*, 964-967

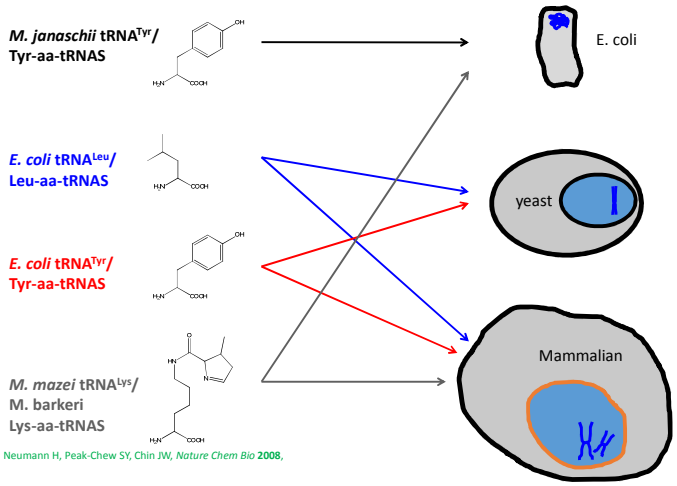
The expanded eucaryotic genetic code

PylRS engineering and the site-specific incorporation of lysine derivatives into proteins in E. coli and mammalian cells.



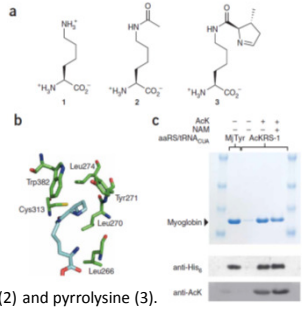
The amino-acid binding pocket with the bound pyrrolysine (X-ray *M. mazei* PylRS•pyrrolysine complex) (**A**), and the structural modeling of the binding pockets of ZLysRS (**B**) and AcLysRS (**C**) bound with Z-lysine and acetyllysine, respectively. (**D**) The space-filling model of Z-lysine in the binding pocket of ZLysRS. (**E**) Production of GST(Am25) containing Z-lysine in *E. coli* cells. (**F**) Production of GRB2(Am111)-FLAG containing Z-lysine in HEK293 c-18 cell. (**G**) The GRB2-FLAG molecules containing Boc-lysine and acetyllysine (CHO and HEK293 c-18 cells). Acetyllysine: 0 mM (−), 1.4 mM (+), and 14 mM (++).

Mukai et al, *Biochem, Biophys Res Com* **2008**, *371(4),* 818-822



Neumann H, Peak-Chew SY, Chin JW, *Nature Chem Bio* **2008**,

Design and evolution of an MbPylRS/tRNA$_{CUA}$ pair for the genetic incorporation of N$^{\varepsilon}$-acetyllysine.



a) Structure of lysine (1),N$^{\varepsilon}$-acetyllysine(2) and pyrrolysine (3).
b) Structure of the active site of *M. Mazei* PylRS bound to pyrrolysine. The active site residues shown are conserved between *M. Mazei* PylRS and *M. Barkeri* PylRS. These residues form the hydrophobic binding pocket of pyrrolysine and are mutated in the library to each of the common 20 amino acids. PDB: 2Q7H.
c) Myoglobin-His 6 produced in the presence of MjTyrRS/MjtRNA$_{CUA}$ (lane 1) or in the presence of AcKRS-1 without or with 1 mM N$^{\varepsilon}$-acetyllysine (AcK, lanes 2 and 3, respectively), or in the presence of 1 mM N$^{\varepsilon}$-acetyllysine and 50 mM NAM (lane 4).

Neumann H, Peak-Chew SY, Chin JW, *Nature Chem Bio* **2008**,

Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome

The *Methanococcus jannaschii* TyrRS–tRNA$_{CUA}$ and the *Methanosarcina barkeri* MbPylRS–tRNA$_{CUA}$ orthogonal pairs have been evolved to incorporate a range of unnatural amino acids in response to the amber codon in *Escherichia coli.*

The general limitation: low efficiency incorporation of a single type of unnatural amino acid at a time, because every triplet codon in the universal genetic code is used in encoding the synthesis of the proteome.

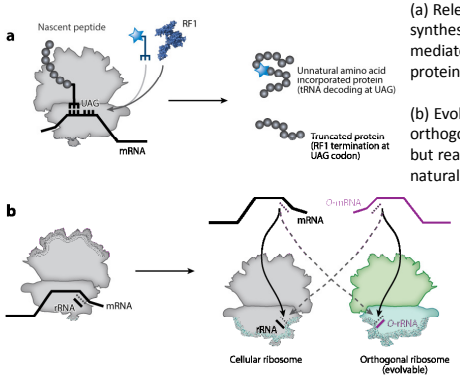
An orthogonal ribosome (ribo-Q1) efficiently decodes a series of quadruplet codons and the amber codon, providing several blank codons on an orthogonal messenger RNA, which it specifically translates. By creating mutually orthogonal aminoacyl-tRNA synthetase–tRNA pairs and combining them with ribo-Q1, incorporation of distinct unnatural amino acids in response to two of the new blank codons on the orthogonal mRNA has been achieved.

It will be possible to encode more than 200 unnatural amino acid combinations using this approach.

The ribo-Q1 independently decodes a series of quadruplet codons

H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin *Nature*, **2010**, *464*, 441-444

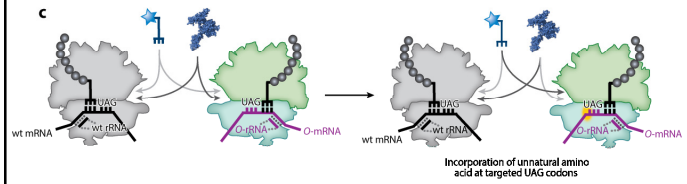
Strategies to enhance unnatural amino acid incorporation in response to the amber stop codon in Escherichia coli.

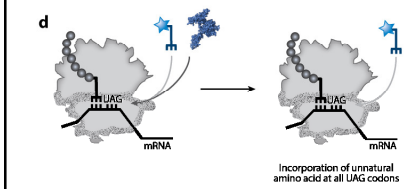


(a) Release factor 1 (RF1)-mediated termination of protein synthesis competes with amber-suppressor transfer RNA (tRNA)-mediated elongation of protein synthesis that yields a full-length protein bearing the unnatural amino acid.

(b) Evolution of an orthogonal ribosome in *E. coli.* The orthogonal ribosome functions alongside the natural ribosome but reads a distinct message that is not a substrate for the natural ribosome.

Strategies to enhance unnatural amino acid incorporation in response to the amber stop codon in Escherichia coli.



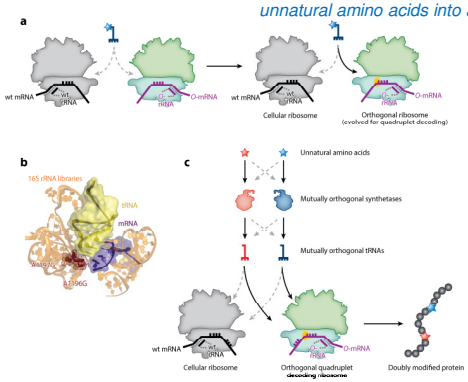


Chin JW. 2014.
Annu. Rev. Biochem. 83:379–408

(c) The orthogonal ribosome has been evolved to efficiently decode amber-suppressor tRNAs, differentiating the decoding of amber codons on the orthogonal and cellular messages and enhancing unnatural amino acids on orthogonal messages without enhancing the incorporation of unnatural amino acids at genomically encoded stop codons.

(d) RF1 knockouts and knockdowns for unnatural amino acid incorporation in E. coli. The strategies increase the incorporation of unnatural amino acids in response to the desired stop codon and any genomically encoded stop codons.

Evolution of an orthogonal quadruplet decoding ribosome enables the incorporation of multiple distinct unnatural amino acids into a single polypeptide.

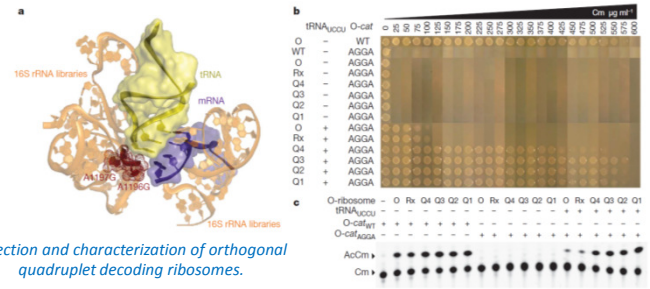


(*a*) The orthogonal ribosome has been evolved in the laboratory to efficiently decode quadruplet codons.

(*b*) Mutations in the A site of 16S ribosomal RNA (rRNA) facilitate quadruplet decoding on the orthogonal ribosome.

(*c*) Genetically encoding multiple unnatural amino acids via orthogonal translation. Mutually orthogonal synthetase/tRNA (transfer RNA) pairs have been used to direct the incorporation of distinct unnatural amino acids into a single polypeptide. The extended anticodon or amber-suppressor tRNAs are selectively decoded on the evolved orthogonal ribosome, creating a parallel translation pathway in the cell.

Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome



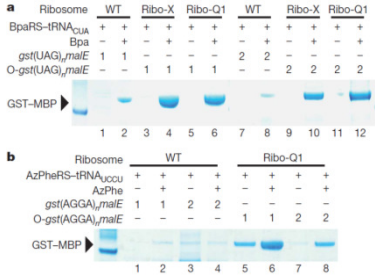
Selection and characterization of orthogonal quadruplet decoding ribosomes.

a) Mutations in quadruplet decoding ribosomes form a structural cluster close to the space potentially occupied by an extended anticodon tRNA. Selected nucleotides are shown in red. **b)** Ribo-Qs substantially enhance the decoding of quadruplet codons. The $tRNA^{Ser}_{UCCU}$-dependent enhancement in decoding AGGA codons in the O-*Cat* (AGGA 103, AGGA 146) gene was measured by survival on increasing concentrations of chloramphenicol (Cm). WT, wild type. **c)** as in **b** , but measuring CAT enzymatic activity directly by thin-layer chromatography.AcCm, acetylated chloramphenicol; O, O-ribosome; Q1–Q4, ribo-Q1–Q4;Rx, ribo-X

H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin *Nature*, **2010**, *464*, 441-444

Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome

Enhanced incorporation of unnatural amino acids in response to amber and quadruplet codons with ribo-Q.



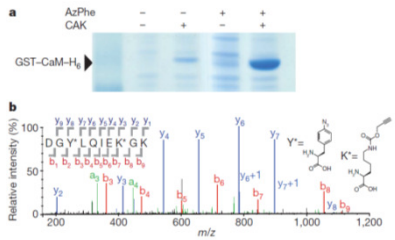
a) Ribo-Q1 incorporates Bpa as efficiently as ribo-X.
b) Ribo-Q1 enhances the efficiency of AzPhe incorporation in response to the AGGA quadruplet codon using AzPheRS*–tRNA$_{UCCU}$.

(UAG)$_n$ or (AGGA)$_n$ describes the number of amber or AGGA codons (n) between gst and malE.

H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin *Nature*, **2010**, *464*, 441-444

Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome

Encoding an azide and an alkyne in a single protein by orthogonal translation

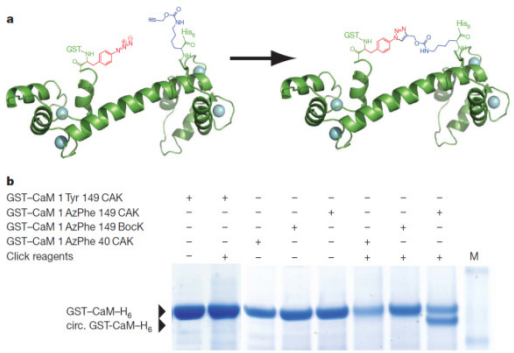


a) Expression of GST–CaM–His$_6$ (a GST–calmodulin–His$_6$ fusion) containing two unnatural amino acids. An orthogonal gene producing a GST–CaM–His$_6$ fusion that contains an AGGA codon at position 1 and an amber codon at position 40 of calmodulin was translated by ribo-Q1 in the presence of AzPheRS*–tRNA$_{UCCU}$ and MbPylRS–tRNA$_{CUA}$.
b) LC–MS/MS analysis of the incorporation of two distinct unnatural amino acids into the linker region of GST–MBP.
Y* - AzPhe; K* - CAK.

H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin *Nature*, **2010**, *464*, 441-444

Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome

Genetically directed cyclization of calmodulin by a Cu(I)-catalysed Huisgen's [2+3]-cycloaddition



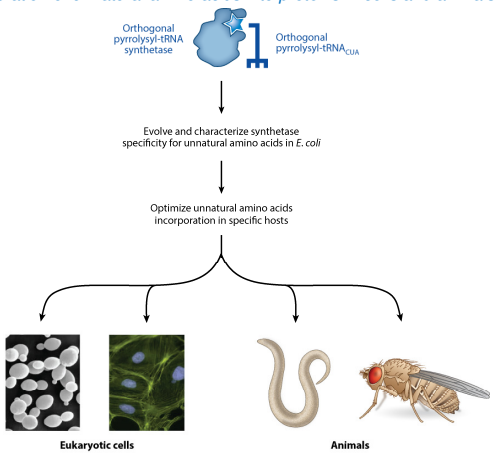
a) Structure of calmodulin indicating the sites of incorporation of AzPhe and CAK and their triazole product.

b) GST–CaM–His_6 1 AzPhe 149 CAK specifically cyclizes with Cu(I)-catalyst.

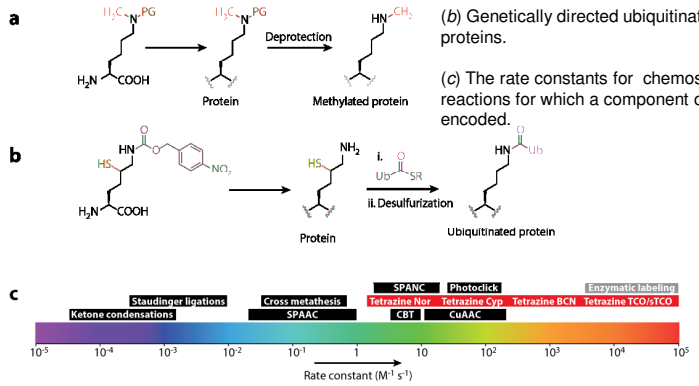
BocK - N^{ε}-*tert*-butyl-oxycarbonyl-*L*-lysine; circ. - circularized protein.

H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin *Nature*, **2010**, *464*, 441-444

The pyrrolysyl-tRNA (transfer RNA) synthetase/$tRNA_{CUA}$ pair can be used to site-specifically encode the incorporation of unnatural amino acids into proteins in cells and animals.



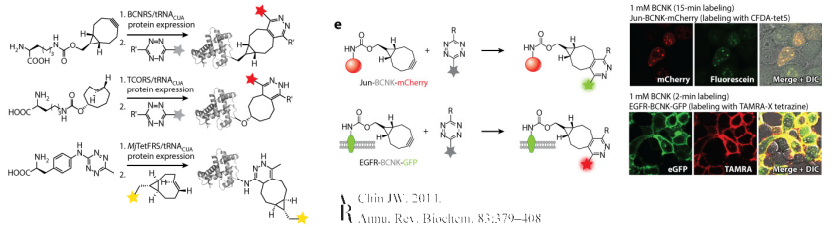
Chemoselective modifications to genetically encoded unnatural amino acids enable both the installation of posttranslationalmodifications that are challenging to directly encode and the rapid labeling of proteins in and on cells.



(*a*) Genetically directed lysine methylation in recombinant proteins.

(*b*) Genetically directed ubiquitination in recombinant proteins.

(*c*) The rate constants for chemoselective (bio-orthogonal) reactions for which a component can be genetically encoded.



(*d*) Components of inverse electron-demand reactions can be genetically encoded in recombinant proteins, facilitating rapid site-specific protein labeling. Lysine derivatives bearing bicyclononynes (BCNs) or *trans*-cyclooctenes (TCOs) are encoded with PylRS/$tRNA_{CUA}$ pair derivatives and labeled with tetrazine probes in rapid and fluorogenic reactions. A tetrazine amino acid has been encoded into *Escherichia coli* by use of a derivative of the *Mj*TyrRS/$tRNA_{CUA}$ pair and labeled with a strained TCO (sTCO) fluorescein derivative.
(*e*) A genetically encoded BCN derivative of lysine (BCNK) allows rapid site-specific protein labeling in and on human cells.

Abbreviations: BCNRS, a pyrrolysyl-tRNA synthetase (PylRS) variant that incorporates a BCN-containing amino acid; CBT, cyanobenzothiazole; CFDA, a cell-permeable fluorescein derivative; CuAAC, copper-catalyzed azide alkyne cycloaddition; Cyp, cyclopropene; DIC, differential interference contrast; eGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; MjTetFRS, a derivative of Methanococcus janaschii tyrosyl-tRNA synthetase (MjTyrRS) that is specific for a tetrazine derivative of phenylalanine; Nor, norbornene; SPAAC, strain-promoted azide alkyne cycloaddition; SPANC, strain-promoted alkyne nitrone cycloaddition; TAMRA, carboxytetramethylrhodamine; TCORS, a PylRS variant for the incorporation of a TCO derivative of lysine; Ub, ubiquitin.