The Role in Translation of Editing and Multi-Synthetase Complex Formation by Aminoacyl-tRNA Synthetases

Dissertation

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ABSTRACT

Aminoacyl-tRNA synthetases (aaRSs) catalyze the first step of translation, aminoacylation. These enzymes attach amino acids (aa) to their cognate tRNAs to form aminoacyl-tRNA (aa-tRNA), an important substrate in protein synthesis, which is delivered to the ribosome as a ternary complex with translation elongation factor 1A (EF1A) and GTP. All aaRSs have an aminoacylation domain, which is the active site that recognizes the specific amino acid, ATP, and the 3′ end of the bound tRNA to catalyze the aminoacylation reaction. Apart from the aminoacylation domain, some aaRSs have evolved additional domains that are involved in interacting with other proteins, recognizing and binding the tRNA anticodon, and editing misacylated tRNA thereby expanding their role in and beyond translation.

One such function of the aaRS is to form a variety of complexes with each other and with other factors by interacting via additional N or C terminal extensions. For example, several archaeal and eukaryotic aaRSs are known to associate with EF1A or other aaRSs forming higher order complexes, although the role of these multi-synthetase complexes (MSC) in translation remains largely unknown. MSC function was hence investigated in the archaeon *Thermococcus kodakarensis*, wherein six aaRSs were affinity co-purified with several other factors involved in protein synthesis, suggesting

that MSCs may interact directly with translating ribosomes. In support of this hypothesis, the aaRS activities of the MSC were enriched in isolated *T. kodakarensis* polysome fractions. These in vivo data indicate that components of the archaeal protein synthesis machinery associate into macromolecular assemblies and could potentially increase translation efficiency by limiting substrate diffusion from the ribosome, thus facilitating rapid recycling of tRNAs.

In addition to their aminoacylation functions, about half of the aaRSs have evolved an editing function, which hydrolyzes non-cognate amino acid from its cognate tRNA thereby maintaining the fidelity of translation. Phenylalanyl-tRNA synthetase (PheRS) misactivates and mischarges Tyr onto $tRNA^{Phe}$, but is able to correct the mistake using a proofreading activity which hydrolyzes mischarged Tyr-$tRNA^{Phe}$. The requirement for PheRS editing and fidelity of Phe codon translation is specific for different cellular compartments in eukaryotes and varies significantly within bacteria. Yeast cytoplasmic PheRS (ctPheRS) has a low Phe/Tyr specificity and is capable of editing, whereas the yeast mitochondrial enzyme (mtPheRS) completely lacks an editing domain, and instead relies on high Phe/Tyr specificity. *Escherichia coli,* in contrast, has retained features of both yeast enzymes and displays a high degree of Phe/Tyr specificity and robust editing activity. We showed that in *E. coli* the editing domain has evolved to efficiently edit *m*-Tyr-$tRNA^{Phe}$, and that this editing activity is essential for cellular growth and viability in the presence of the non-proteinogenic amino acid *m*- Tyr and oxidative stress conditions. In comparison, in the yeast enzyme, due to the low specificity of its active site, cytoplasmic PheRS editing has evolved to protect the proteome from *p*-

Tyr misincorporation as shown by the requirement for editing activity to survive in the presence of high concentrations of Tyr compared to Phe. Hence different environmental factors and cell physiology drive the selection of quality control mechanisms in various organisms.

While PheRS has evolved to possess editing activity to actively edit noncognate amino acids (both proteinogenic and non-proteinogenic), editing mechanisms are not evolutionarily conserved. Tyrosyl-tRNA synthetases are among the aaRSs lacking any known editing activity. The high specificity displayed by this aaRSs is achieved by taking advantage of the unique structural and chemical properties of certain amino acids, leading to favorable binding affinities of cognate over non-cognate substrates in the active site of the enzyme. Its cognate amino acid Tyr differs from Phe by a single hydroxyl group, and the specific recognition and binding of the hydroxyl group allows bacterial TyrRS to discriminate against non-cognate Phe with a specificity of 10^5. However, recent studies have suggested that error rates may actually vary considerably during translation under different growth conditions. We examined the misincorporation of Phe at Tyr codons during synthesis of a recombinant antibody produced in tyrosine-limited Chinese hamster ovary (CHO) cells. Tyr to Phe replacements were found to occur throughout the antibody at a rate of up to 0.7% irrespective of the identity or context of the Tyr codon translated. Monitoring of Phe and Tyr levels revealed that changes in error rates correlated with the decrease of Tyr in the amino acid pools, suggesting that mischarging of $tRNA^{Tyr}$ with non-cognate Phe by TyrRS was responsible for mistranslation. Steady-state kinetic analyses of CHO cytoplasmic TyrRS revealed a twenty five-fold lower

specificity for Tyr over Phe compared to previously characterized bacterial enzymes, consistent with the observed increase in translation error rates during tyrosine limitation. Functional comparisons of mammalian and bacterial TyrRSs revealed key differences at residues responsible for amino acid recognition, highlighting differences in evolutionary constraints for translation quality control.

DEDICATION

This document is dedicated to my family especially my father Vijay Raina, mother Kusum Raina, my sister Sneha Raina and my husband Sharat Menon for their unconditional love, support and constant encouragement throughout my education.

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FIELDS OF STUDY

Major Field: Biochemistry

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LIST OF SYMBOLS AND ABBREVIATIONS

2-Abu	2-aminobutyrate
4-hPro	4-hydroxyproline
γ-hLeu	γ-hydroxyleucine
Å	angstrom (unit)
aa	amino acid
aaRS	aminoacyl-tRNA synthetase
aa-tRNA	aminoacyl-tRNA
ADP	adenosine 5'-diphosphate
AIMP	aaRS-interacting multifunctional protein
Ala	L-alanine
AMP	adenosine 5'-monophosphate
Arg	L-arginine
AS	active site
ASD	anti-shine-dalgarno
Asn	L-asparagine
Asp	L-aspartic acid
ATP	adenosine 5'-triphosphate

β	beta
BSA	bovine serum albumin
°C	degrees Celsius (centigrade)
ct	cytoplasmic
CHO	chinese hamster ovary
CP1	connective peptide 1
Cys	L-cysteine
Δ	delta/deletion
DDT	dithiothreitol
DNA	deoxyribonucleic acid
Ec	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EF1A	elongation factor 1 alpha
EF-Tu	elongation factor Tu
g	gram
GDP	guanosine 5′-diphosphate
Gly	L-glycine
GTP	guanosine 5’ triphosphate
Gln	L-glutamine
Glu	L-glutamic acid
Gly	L-glycine
GTP	guanosine 5′-triphosphate

h	hour
Hcy	homocysteine
HEPES	N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]
His	L-histidine
Hse	homoserine
IF	initiation factor
Ile	L-isoleucine
INS	insertion domain
IPTG	isopropyl-β-D-thiogalactoside
k_{cat}	catalytic rate
K_M	Michaelis constant
LB	Luria-Bertani
Leu	L-leucine
Lys	L-lysine
µ	micro
µL	micro litre
µM	micro molar
M	molar
Met	L-methionine
min	minute
mL	milli litre
mM	milli molar

MM	minimal media
mRNA	messenger RNA
MSC	multi-synthetase complex
mt	mitochondrial
nm	nanometer
nLeu	L-norleucine
Orn	L-ornithine
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Phe	L-phenylalanine
PP_i	inorganic pyrophosphate
Pro	L-proline
RBS	ribosome binding site
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
Sc	*Saccharomyces cerevisiae*
SD	shine-dalgarno
Ser	L-serine
SerHX	serine hydroxymate
SMM	supplemented minimal media

TCA	trichloroacetic acid
Thr	L-threonine
TLC	thin-layer chromatography
Tris-HCl	tris-(hydroxylmethyl) aminomethane hydrochloride
tRNA	transfer RNA
Trp	L-tryptophan
Tyr	L-tyrosine
Ura	uracil
UV	ultra violet
Val	L-valine
WT	wild-type
YPDA	yeast peptone dextrose agar

Chapter 1:

Introduction

1.1 Protein synthesis

Protein synthesis is one of the important steps in gene expression, by which the information encoded as nucleic acids in mRNA is finally translated into the amino acids that make up proteins. The fidelity of protein synthesis depends on highly specific attachment of amino acids (aa) to tRNAs during aminoacyl-tRNA (aa-tRNA) synthesis and precise mRNA:tRNA decoding interactions on the ribosome. Aminoacyl-tRNA synthetases (aaRSs) play an essential role in protein synthesis by attaching the correct amino acid onto the cognate tRNA. Once formed, aa-tRNAs are bound by elongation factors (EF1A in eukaryotes and archaea; EF-Tu in bacteria) and are delivered to the ribosome in a ternary complex EF1A·GTP·aa-tRNA, providing substrates for translation elongation.

AaRSs are modular enzymes composed of conserved catalytic cores and additional appended domains acquired during evolution (1, 2). These domains are

required for RNA recognition and proofreading activities and are also involved in protein–protein interactions and various other roles, thereby expanding aaRS functions beyond translation of mRNA. Another important substrate in protein synthesis is tRNA. Apart from their role as adaptors during translation of the genetic code, tRNAs also perform additional functions in both prokaryotes and eukaryotes for example in regulating gene expression. Aminoacylated tRNAs have also been implicated as substrates for non-ribosomal peptide bond formation, post-translational protein labeling, modification of phospholipids in the cell membrane and antibiotic biosyntheses. Most recently tRNA fragments, or tRFs, have also been recognized to play regulatory roles. Here we examine in more details the various functions of aaRS in translation and beyond and also some of the new functions emerging for tRNA in a variety of cellular processes outside of protein synthesis.

1.2 Aminoacyl-tRNA synthetases.

1.2.1 Aminoacylation domain architecture and aminoacylation activity

Aminoacylation is a two-step reaction catalyzed by the aminoacylation domain, which encodes the active site in all aaRSs. The first step is activation of an amino acid with ATP to form aminoacyl adenylate and the second step involves the transfer of the aminoacyl moiety to the 3’ end of the tRNA, thereby forming aa-tRNA (Fig. 1.1) (3). Although all aaRSs catalyze similar aminoacylation reactions, they can be divided into two classes (Class I and II) based on their active site architecture and mode of tRNA binding, except

for lysyl-tRNA synthetase (LysRS), which has a representative in both classes. Each class is further subdivided into subclasses a, b, and c on the basis of relative sequence and structural similarity. Class I aaRSs contain a Rossmann nucleotide-binding fold and include the highly conserved signature sequences KMSKS and HIGH. AaRSs in this class are usually monomeric except TyrRS, TrpRS and MetRS which are all homodimers. The Rossmann fold binds ATP and amino acid and promotes catalysis. In contrast, class II aaRS active sites are composed of a seven-stranded β-sheet with flanking α-helices consisting of motifs 1, 2, and 3. Motifs 2 and 3 aid in binding ATP and amino acid, while motif 1 is important for dimerization. Class II aaRSs are mostly dimeric or multimeric (4, 5). The other difference between the two classes highlights their different modes of binding to tRNA. Class I enzymes approach tRNA from the minor groove of the tRNA acceptor stem (except TyrRS), aminoacylating the terminal adenosine of the tRNA molecule at the 2'-OH position, while class II aaRSs approach the major groove of their respective tRNAs and couple the amino acid to the 3'-OH of the terminal adenosine except PheRS, which utilizes the 2'OH (5-7).

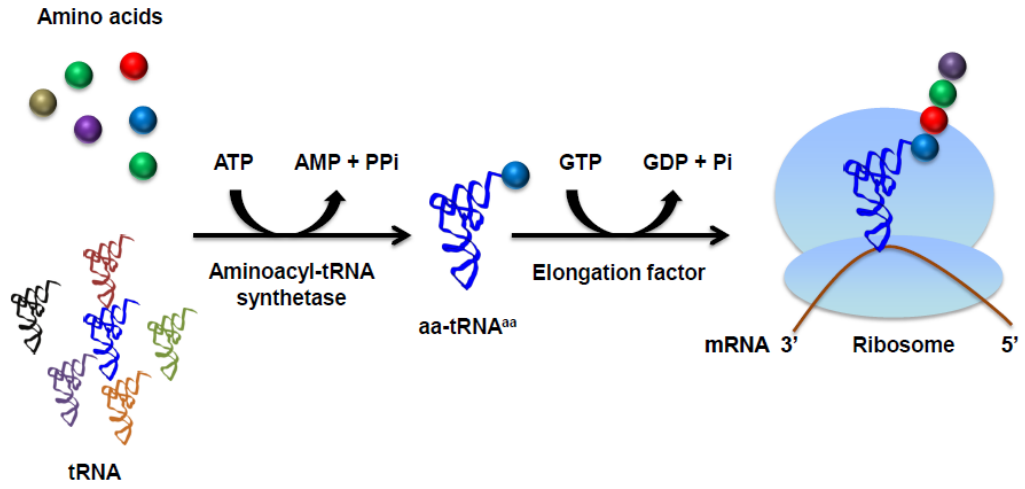


Figure 1.1. Schematic of aminoacylation reaction and co-translational insertion of tRNA

1.2.2 Specificity of aaRS active site

The overall error rate of translation is generally believed to be 10^{-4} (8, 9), with the first step, synthesis of aa-tRNA, being the most error prone. The error rate of this first step of translation is largely dependent on the specificity of the aaRS, that is selection of the correct amino acid and tRNA from the cellular pool of predominantly non-cognate substrates. AaRSs select their cognate tRNAs by exploiting sequence specific differences between various tRNAs during binding and aminoacylation thereby resulting in a low error rate of 10^{-6} at this step (6, 10). The specificity of recognition of cognate tRNAs by aaRSs is achieved by the presence of tRNA identity elements (6). tRNA identity elements consists of both positive (determinants) and negative (antideterminants) sequence elements, which promote correct interaction between the cognate tRNA and aaRS and

prevent mischarging of noncognate tRNA substrates, respectively. The identity elements are conserved among most organisms and are located predominantly at the two distal ends of the tRNA, in the acceptor stem that contains the important discriminator nucleotide N73, and in the anticodon loop. However, there are unique identity elements that are specific to certain tRNAs like the G3:U70 wobble base pair of $tRNA^{Ala}$ (11-14), G15:G48 Levitt pair of $tRNA^{Cys}$ (15, 16) and G^{-1} base of $tRNA^{His}$ (17-19). Some modified nucleotides of tRNA mostly located in the anticodon loop are also known to contribute to the specificity of aaRS-tRNA interactions (6).

In contrast, selection of the correct amino acid is often challenging due to the lack of sufficient discriminating functional groups in many amino acids and their analogs. In order to maintain the low error rate of translation, editing mechanisms have evolved to discriminate between substrates with close structural and chemical properties by hydrolyzing either the activated non-cognate amino acid (pre-transfer editing) or mischarged tRNA (post-transfer editing) (20). The high specificity displayed by some aaRSs is achieved by taking advantage of the unique structural and chemical properties of certain amino acids, leading to favorable binding affinities of cognate versus non-cognate substrates in the active site of the enzyme. For example, Phe and Tyr differ from each other only by a single hydroxyl group, posing a threat to PheRS specificity and hence the enzyme has evolved an editing domain to clear mischarged Tyr, However, bacterial tyrosyl-tRNA synthetase (TyrRS) can discriminate against non-cognate Phe with a specificity of 10^5 by specific recognition and binding (21).

1.2.3 Editing mechanisms

To minimize error rates in protein biosynthesis, both classes of aaRSs have evolved several hydrolytic proofreading activities (22, 23). Mistakes are eliminated before protein synthesis by aaRS activities that hydrolyze misactivated aminoacyl-adenylates (pre-transfer editing) and/or mischarged aa-tRNAs (post-transfer editing). Pre-transfer editing occurs immediately following ATP-dependent activation of a non-cognate amino acid, before it is transferred to tRNA (Fig. 1.2). It can be either tRNA dependent or independent, and occurs *via* multiple pathways including tRNA independent release of the misactivated noncognate amino acid followed by spontaneous hydrolysis in solution, tRNA independent hydrolysis, cyclization of the aminoacyl adenylate in the active site, or tRNA dependent hydrolysis (22, 23). An example of an aaRS exhibiting tRNA-independent enzymatic pre-transfer hydrolysis is Class I MetRS. MetRS has been shown to activate noncognate homocysteine (Hcy), which is a smaller naturally occurring precursor in the methionine biosynthetic pathway and differs from Met by a single methyl group. MetRS eliminates activated Hcy by catalyzing intramolecular cyclization of Hcy in the active site forming the nonproductive homocysteine thiolactone, which cannot participate in the second tRNA transfer step (24). Other aaRSs using tRNA independent pre transfer mechanisms for editing include Class II LysRS against homoserine (Hse) and ornithine (Orn) (25), ProRS against Ala (26) and SerRS against Thr, Cys and Ser-HX (27). A unique tRNA dependent pre-transfer editing pathway has been elucidated for Class I IleRS, which synthesizes non-cognate Val-AMP. Upon activation of Val, Ile mischarges Val onto $tRNA^{Ile}$. The Val-$tRNA^{Ile}$ thus formed causes a

conformational change in the enzyme complex, which triggers translocation of the mischarged amino acid to the editing site, where it is immediately hydrolyzed. However, the 3' end of the tRNA presumably remains bound and in this editing active state the complex is primed for pre-transfer editing should another misactivation event occur (28). tRNA independent pre-transfer editing mechanisms are also employed by Class II AlaRS (29) and PheRS (30), in addition to their post-transfer editing mechanisms against misacylated tRNAs. The presence of both pre and post-transfer editing mechanism in some aaRS raised questions regarding the partitioning between these two mechanisms. However a study of yeast cytoplasmic LeuRS showed that the partitioning between the two editing mechanism is dependent on the identity of the misactivated noncognate amino acids. While LeuRS efficiently deacylates mischarged Ile-tRNA^{Leu} via post-transfer editing mechanism, it shows poor activity against Met-tRNA^{Leu} and employs enzyme-mediated pre-transfer editing to clear the misactivated methionyl-adenylate (31).

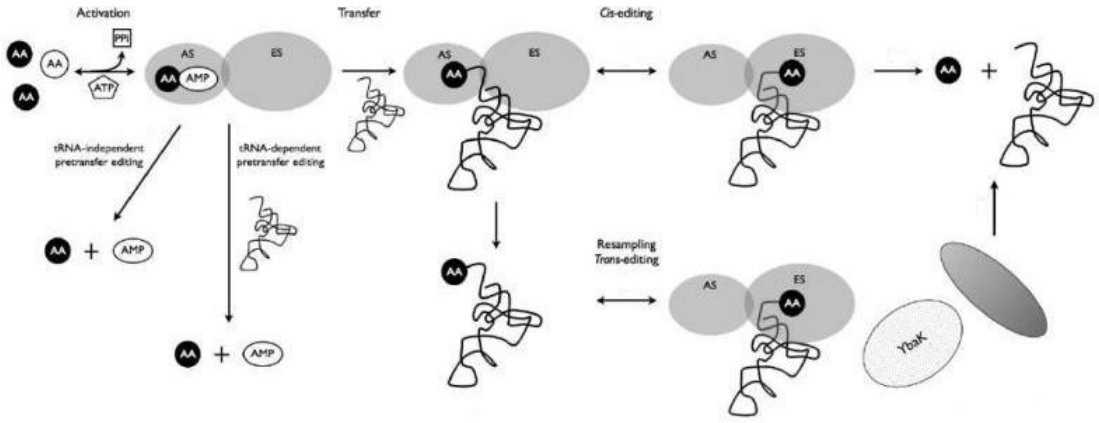


Figure 1.2. Fate of mischarged tRNAs in protein synthesis. Misactivated amino acids can be edited prior to their transfer to the tRNA either in a tRNA independent or dependent fashion. Following transfer mischarged aa-tRNA can be edited in *cis* or *trans* by the aaRS editing site. Free-standing editing factors such as YbaK and AlaXps can also hydrolyze mischarged aa-tRNAs. Adapted from (32).

Following aminoacylation of tRNA with a non-cognate amino acid, several aaRSs utilize a post-transfer editing mechanism to hydrolyze mischarged aa-tRNAs and prevent amino acid misincorporation during translation. The post-transfer editing reaction is carried out by a distinct editing site site, 35–40 A° away from the aminoacylation site. The first model for post transfer editing, the double–sieve editing model, was proposed by Alan Fersht to explain the proofreading mechanism of aaRSs. According to the model, the active site acts as the first sieve excluding larger amino acids from entering the active site meanwhile allowing the cognate and smaller amino acids to bind and be activated. A second, fine sieve located in the editing site is then responsible for selectively hydrolyzing misacylated tRNAs, while excluding the correctly aminoacylated tRNAs by taking advantage of size and chemical properties of the amino acid (33). Fersht *et al.*

further demonstrated this model for Isoleucyl-tRNA synthetase (IleRS), which misactivates Valine only 200-fold less efficiently than its cognate amino acid Isoleucine. Amino acids larger than the cognate Ile are excluded from the first sieve, $tRNA^{Ile}$ mischarged with smaller noncognate amino acids are hydrolyzed, while Ile-$tRNA^{Ile}$ is excluded and released for protein synthesis (34).

The double sieve mechanism helps maintain a low error rate for the highly error-prone aminoacylation step of ~ 1 in 3000, which is compatible with an error rate of translation of 10^{-4} (35). Post-transfer editing by aaRSs, can occur either in *cis* or in *trans*. An important feature of the post-transfer editing reaction is movement of the 3′-CCA end of the mischarged tRNA from the aminoacylation site into the editing site (32). The *cis* mechanism of post-transfer editing involves direct translocation of the 3′-CCA end of the tRNA from the aminoacylation site to the editing site. Support for the *cis* model of editing comes from various structural studies of class I aaRSs and also from studies showing that aa-tRNA release is the rate-limiting step in aminoacylation for class I aaRSs, giving the 3′-CCA time to move from the aminoacylation site to the editing site (36-38). In contrast, the *trans* mechanism involves the release of the mischarged tRNA from the active site after aminoacylation, followed by rebinding of the aa-tRNA to the aaRS at the editing site (39, 40). The *trans* model of post-transfer editing is supported by the study of Ling *et al.* showing resampling of mischarged aa-tRNA by the editing domain of an aaRS and also studies showing that aa-tRNA release during the aminoacylation reaction is not rate-limiting and aa-tRNAs can rapidly release and rebind to aaRS (41). Both class I and class II aaRSs use post-transfer editing activities to get rid

of mischarged aa-tRNAs. Examples of aaRSs with this activity include IleRS (42), ValRS (34), LeuRS (43), PheRS (44), ThrRS (45), ProRS (46), and AlaRS (Table 1.1) (29).

Post-transfer editing activity is not confined to the editing domains of aaRS. Many freestanding editing factors are found in all three domains of life. These factors act in *trans* to clear mischarged aa-tRNAs (22). Examples of *trans* editing factors include D-Tyr-tRNA^{Tyr} deacylases (47), AlaXps (48), and YbaK (49) which trans-edit D-Tyr-tRNA^{Tyr}, Ser- tRNA^{Ala} and Cys-tRNA^{Pro}, respectively. In the specific case of tRNA^{Pro}, *trans*-editing by YbaK constitutes an additional layer of quality control for ProRS. The ProRS insertion domain (INS) *cis*-edits mischarged Ala-tRNA^{Pro} (46) whereas YbaK in complex with ProRS serves to *trans*-edit Cys-tRNA^{Pro} (49, 50). Four other proteins homologous to the INS domain of ProRS belonging to the Ybak superfamily are ProX, PrdX, PA2301 and YeaK. PrdX has been shown to edit Ala-tRNA^{Pro}; however, the substrates of the other homologues are not known (51). How editing functions have become associated with the aaRS in some instances while in other cases the editing domains have remained independent of the aaRS is not fully understood, although it may reflect the need to edit a wider range of non-cognate species for particular tRNA isoacceptors.

Table 1.1. Known mode of editing and amino acid substrates of aminoacyl-tRNA synthetases. Adapted from (23).

Class	aaRS	Structural organization	*Cis*-editing domain	*Trans*-editing factor	Amino acids misactivated
Class I	IleRS	α	**CP1, AS**	-	**Val, Hcy, Leu, Cys, Thr, 2-Abu**
	LeuRS	α	**CP1, AS**	-	**Val, Ile, Met, Hcy, γ-hLeu, nLeu**
	ValRS	α	**CP1**	-	**Thr, 2-Abu, Hcy, Cys**
	MetRS	α_2, α	**AS**	-	**Hcy**
Class II	ThrRS	α_2	**N2**	**ThrRS-ed**	**Ser**
	ProRS	α_2	**INS, AS**	**YbaK superfamily**	**Ala, Cys, 4-hPro, 2-Abu**
	PheRS	$(\alpha\beta)_2$, α	**B3/B4**	-	**Tyr, Ile, *m*-tyr**
	AlaRS	α_4, α	**C-terminal, AlaX-like**	**AlaXp**	**Gly, Ser**
	SerRS	α_2	**AS**	-	**Thr, Cys, Ser-HX**
	LysRS II	α_2	**AS**	-	**Hcy, Hse, Orn**

1.2.4 Role of specificity of aaRS active site and editing in the cell

Translation accuracy is vital for the maintenance of cellular integrity. Accuracy in protein synthesis is dependent on a combination of sequential substrate recognition events, which include the synthesis of correct aminoacyl tRNAs (aa-tRNA) by aminoacyl tRNA synthetases (aaRS), binding of (EF1A) to the cognate aa-tRNA and the selection of the correct aa-tRNA by the ribosome. All these steps have their own inherent error rate which is thought to vary depending on various environmental conditions. The error rate of the aminoacylation reaction, is largely dependent on the specificity of the aaRS, that is selection of the correct amino acid and tRNA from the respective cellular pools of predominantly non-cognate substrates (6, 10). In contrast, selection of the correct amino acid is often challenging due to the lack of sufficient discriminating functional groups in many amino acids and their analogs. In order to maintain a low error rate during translation, editing mechanisms have evolved to discriminate between substrates with close structural and chemical properties by hydrolyzing either the activated non-cognate amino acid (pre-transfer editing) or mischarged tRNA (post-transfer editing). These quality control mechanisms help maintain an error rate in protein synthtesis of around 1 in 10,000 (35). However, quality control mechanisms have been shown to be dispensable to the cell under normal growth conditions. Recent studies suggest that error rates vary considerably according to different environmental conditions. In *E. coli*, codon-specific differences in error rates of up to 18-fold were observed using a luciferase reporter assay (9). More dramatically, exposure of mammalian cells to a variety of stresses elevates tRNA mischarging to levels that could potentially lead to increases in the error rate of

translation of 100-fold or more for some codons (52, 53). Hence under various cellular stresses like nutrient deprivation, where the concentration of non-cognate amino acid might increase compared to cognate, and other stress conditions like oxidative stress, which are likely to increase the error rate of translation, quality control may become essential to cells. For example, editing-defective AlaRS from mouse fibroblast (54) and an archaeal *Sulfolobus solfataricus* editing-deficient strain (55) become sensitive to exogenous addition of excess non-cognate serine as observed by slow growth and reduced viability. Cells expressing editing deficient aaRSs have been shown to exhibit increased sensitivity to antibiotics that inhibit DNA replication, ribosomal function, or cell wall synthesis thereby displaying a slow growth phenotype (56, 57).

Quality control mechanisms have evolved differently in different organisms. *S. cerevisiae* cytoplasmic PheRS (*Sc*ctPheRS) displays a relatively low specificity for its cognate amino acid Phe compared to non-cognate amino acid Tyr and hence relies on a post-transfer editing activity to clear mischarged $tRNA^{Phe}$, whereas the yeast mitochondrial enzyme (*Sc*mtPheRS) completely lacks an editing domain, and instead relies on its high Phe/Tyr specificity. In contrast *E. coli* PheRS exhibits both high specificity towards Phe and a robust editing activity (58). In some organisms, like *Mycoplasma mobile,* aaRSs including PheRS have evolved to lack editing function, and are capable of tolerating the accumulation of errors in their proteome (59, 60). The diverse evolution of different quality control mechanisms in different organism together with the dispensability of these mechanisms indicate that the true roles of these quality control pathways are not fully understood.

1.2.5 Aminoacyl-tRNA containing multi-enzyme complexes

AaRSs are modular enzymes composed of conserved catalytic cores and additional appended domains acquired during evolution (2). One of the most important roles of appended domains in higher eukaryotic and some archaeal aaRSs is to mediate the formation of intricate networks of protein–protein interactions between different components of the translation machinery and other components of the cell, thereby expanding the functions of aaRSs. Although many of these associations were first described in eukaryotic cells, numerous multi-enzyme complexes containing aaRSs have recently been identified in both Bacteria and Archaea. In mammalian cells, among the 20 aaRSs, 9 (ArgRS, AspRS, GlnRS, GluProRS, IleRS, LeuRS, LysRS and MetRS) associate with three non-synthetase protein factors p18 (AIMP1), p38 (AIMP2), and p43 (AIMP3) to form a multi-synthetase complex (MSC) (61). Based on low resolution electron microscopic images this large 1.4 MDa complex is thought to assemble into a V – shaped complex (62, 63). AspRS, MetRS, and GlnRS form one arm, while the other arm is made up of LysRS and ArgRS. The bifunctional GluProRS, IleRS, and LeuRS form the base of the V-shaped macromolecule (64). Interaction of aaRSs within the MSC are mediated via protein-protein interactions through either N- or C-terminal appended eukaryotic domains, as observed for MetRS, LeuRS, and ArgRS (65, 66), or via their catalytic domains (LysRS, AspRS, and GlnRS) (67). AIMP2 is thought to be an essential scaffold molecule for the assembly of the MSC, with GlnRS, ArgRS, AIMP1 interacting with the N-terminal region of AIMP2 and LysRS, MetRS, AspRS, GluProRS, IleRS, LeuRS and AIMP3 interacting with the C-terminal region of AIMP2 (68). The three

accessory proteins are important for the formation and stability of the complex, promote binding of tRNAs to the complex, and also play other roles outside translation (69-71). In addition to the large MSC, a smaller complex of ValRS and EF-1H has also been reported. ValRS interacts with the EF-1H complex via its N terminal domain and this interaction has shown to increase the aminoacylation activity of ValRS (72).

In lower eukaryotes like yeast, the MSC is composed of two aaRSs GluRS, MetRS and the non-enzyme component Arc1p, which is homologous to mammalian AIMP1 (73). Arc1p binds to the N-terminal domains of both GluRS and MetRS and facilitates the binding of tRNA to the two aaRS along with regulating the subcellular localization of the two aaRS (74). Apart from the GluRS-MetRS-Arc1p complex, a complex between TyrRS and Knr4P, a protein involved in cell wall biosynthesis, and a SerRS-Pex21p complex are also observed in *S. cerevisiae*. Pex21p is a protein involved in peroxisome biosynthesis that binds to the C terminus of SerRS and has been shown to promote binding of $tRNA^{Ser}$, thereby enhancing the activity of SerRS (75, 76).

AaRS complexes have also been reported in archaea with the first complex described in *Haloarcula marismortui*, with many if not all of the aaRSs purified in one or possibly two large complexes (77, 78). In the archaeal methanogen *Methanothermobacter thermautotrophicus*, a complex composed of LeuRS, LysRS, ProRS and EF1A, improves the catalytic efficiency of tRNA aminoacylation by both LysRS and ProRS (79-82). In the same organism, interactions between the atypical form of SerRS confined to certain archaea (mSerRS) and ArgRS, and between mSerRS and the ribosomal protein L3 have also been identified (83). Complex formation was shown to increase the activity of

mSerRS induced by ArgRS under conditions of elevated temperature and osmolarity. The complex between *M. thermautotrophicus* SerRS and ArgRS provides a means by which the methanogenic archaea can optimize the aminoacylation reaction under a wide range of extreme environmental conditions.

1.2.6 Role of MSC in translation

To date the role of MSCs in translation is not clearly understood. The presence of only some of the aaRS among the 20 in the MSC raises questions about the selection pressure that determines these interactions. The aaRSs in the MSC are not selected based on their activities or active site structure as demonstrated by the presence of both class I and class II aaRSs in the complex. However, it has been observed that the aaRSs activating hydrophobic and non-aromatic amino acids are present within the complex, while those aminoacylating the smallest and largest amino acids are absent (84). MSCs contain various tRNA binding domains like the p43 dimer, the R domains of GluProRS and MetRS and the K domains of AspRS and LysRS dimers which exhibit strong tRNA binding potential that might be shared by other aaRSs in the complex, thereby increasing the efficiency of their respective aminoacylation reaction. The interaction of aaRSs with other proteins in the complex has also shown to increase aminoacylation efficiencies. For example, in humans, EF1A associates with the guanine nucleotide recycling machinery, EF-1βδγ, to form the larger elongation factor 1 H (EF-1H) complex, which in turn strongly interacts with ValRS via the N-terminal extension of this aaRS. The presence of EF1A-GTP was shown to increase the catalytic efficiency of aminoacylation by two fold.

This increase was not observed in the presence of EF1A in the GDP, form indicating that the increased levels of Val-tRNA^{Val} formed were due to improved catalysis by ValRS, and not the protective effects of EF1A (85). The product release step in the aminoacylation reaction is rate limiting in class I enzymes, hence it could be possible that class I aaRSs interact with EF1A or class II aaRS to overcome this rate limiting step thereby increasing the efficiency of aminoacylation (38). EF1A has also been observed to associate with a class II aaRS, AspRS, which stimulated the rate-limiting step of Asp-tRNA^{Asp} release (86). Increased catalytic efficiency of aaRSs has also been observed in the archaeal MSC. In *M. thermautotrophicus*, the MSC is comprised of three aaRS (LeuRS, LysRS and ProRS) and EF1A. The interaction between LeuRS and EF1A was shown to significantly increases the k_{cat} for Leu-tRNA^{Leu} synthesis (79), in agreement with the proposal that EFs may be predisposed to form complexes with those aaRSs that are rate-limited by aa-tRNA release (38). Also, it was discovered that in the presence of LeuRS, the catalytic efficiencies of aminoacylation by LysRS and ProRS were enhanced, suggesting the role of MSC in translation might be to enhance the aminoacylation by the associated aaRS (79, 80).

Complex formation between the elongation factor and an aaRS leads to direct channeling of aminoacyl-tRNAs from aaRS to the site of protein synthesis by the transfer of aa-tRNA directly from the aaRS to EF1A without dissociation into the cytoplasm (87). The channeling model or tRNA cycle was first proposed by Smith in 1975 (88). This channeling model is supported by the fact that endogenous aa-tRNAs are protected from degradation by RNAses as compared to the exogenous aa-tRNAs, and exogenous aa-

tRNAs introduced into the cell cannot take part in protein synthesis (89). According to the proposed channeling scheme, aa-tRNAs are vectorially transferred from aaRSs to ribosomes as tertiary complexes of EF1A, GTP and aa-tRNA (89, 90). It has been suggested that the deacylated tRNA at the Exit (E) site of ribosomes is recycled to the aaRSs either by binding to the GDP-bound form of EF1A (91) or directly to the aaRSs (90), thereby providing a sequestered pool of aa-tRNAs specifically used in protein synthesis (tRNA cycle). This model is further supported by the presence of two ArgRSs in the mammalian cells, full length ArgRS, which is complexed with other aaRSs in the mammalian MSC via an appended N-terminal domain, and a free form which has a truncated N-terminus. The two forms of ArgRS are translated from alternative start codons (92). It was observed that the pool of aa-tRNAs synthesized by ArgRS complexed with the MSC were preferentially utilized as substrates for protein synthesis *in vivo* compared to externally added aa-tRNA or uncharged tRNA (93). This study thereby supports the channeling model, in which mammalian MSC is intimately involved in protein synthesis by providing the elongating ribosome with substrates synthesized within the MSC (85, 89).

Apart from increasing the efficiency of translation, MSCs have also been hypothesized to serve as depots to regulate cellular localization and the release of various components of the complex, that upon, release acquire new functions in the cell (94). The bifunctional GluProRS, which harbors two different aaRS catalytic activities separated by three tandem linker domains, lends support to this hypothesis. GluProRS is phosphorylated by IFN-γ which induces release of GluProRS from the MSC. Once

released, the phosphorylated GluProRS forms IFN- γ activated inhibitor of translation (GAIT) complex composed of GluProRS, NSAP1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ribosomal protein L13a. The active GAIT complex then binds to the GAIT element in the 3' UTR of the target mRNA and inhibits its translation by blocking ribosome recruitment (94, 95). Further studies are required to understand the selection pressure for aaRS to form such complexes. Also, further investigation of the interaction of all the components proposed to be part of the tRNA cycle is required to help elucidate how MSCs affect translation.

1.3 Transfer RNA (tRNA)

tRNAs are important players in the protein synthesis pathway, linking the genetic code with the amino acid sequence of proteins. tRNAs are comprised of 73–90 nucleotides and have a characteristic cloverleaf secondary structure made up of the D-loop, T loop, variable loop and the anticodon loop. The tRNA further folds into an L-shaped tertiary structure through coaxial stacking of the T and D loop. To function as a substrate in protein synthesis tRNA is charged with an amino acid by its cognate aminoacyl-tRNA synthetases. The aminoacyl-tRNA (aa-tRNA) thus formed serves as a substrate and participates in the chemistry of peptide bond formation in the process of protein synthesis. Beside this well-known canonical role during protein biosynthesis, tRNAs perform additional functions such as acting as signaling molecules in the regulation of numerous metabolic and cellular processes in both prokaryotes and eukaryotes. Aminoacylated tRNAs have also been implicated as substrates for non-ribosomal peptide

bond formation in the case of cell wall formation, protein labeling for degradation, modification of phospholipids in the cell membrane and antibiotic biosynthesis. Due to their universally conserved L-shaped three-dimensional conformation, which is stabilized by extensive secondary and tertiary structural contacts and modifications, tRNA molecules are among the most stable RNAs in a cell and are considerably more robust than mRNAs (96). For a long time tRNA fragments were considered as non-functional degradation intermediates, but have now been recognized to be major RNA species in human cells for which regulatory roles are beginning to be discovered. It was also recently shown that tRNAs can act as an effective scavenger of cytochrome c, consistent with a role in regulating apoptosis. With new functions still emerging for tRNA, we examine some of the many ‘‘non-protein synthesis” roles of tRNA in the cell (Fig. 1.3).

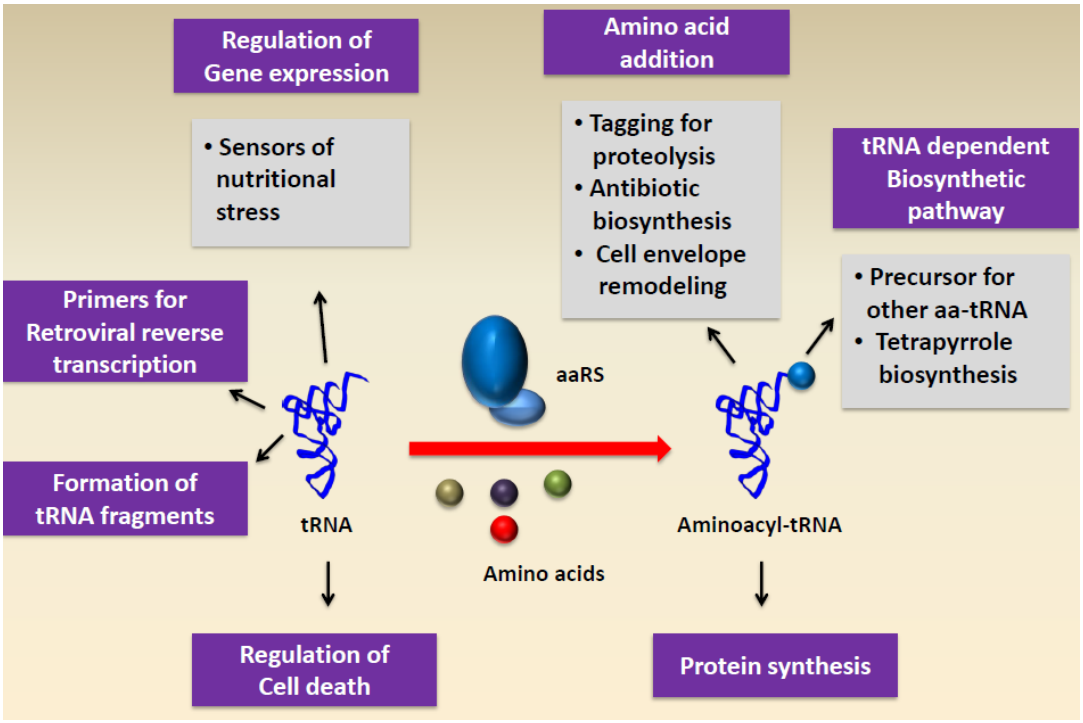


Figure 1.3. Various roles of charged and uncharged tRNA in the cell.

1.3.1 Roles of tRNA in gene expression

While aa-tRNAs have been implicated in many roles outside translation several important functions of tRNA do not require the aminoacyl form. Uncharged tRNAs have been shown to regulate global gene expression in response to changes in amino acid pools in the cell. Bacteria have adopted various strategies to adapt to external stresses, of which the most-studied global regulatory systems is the stringent response. Stringent response is mediated through the production of the alarmone 5'-diphosphate 3'-diphosphate guanosine (ppGpp) and 5'-triphosphate 3'-diphosphate guanosine (pppGpp), which were discovered in 1969 by Cashel and Galant in *E. coli* as a response to amino acid starvation

(97). *E. coli* uses two pathways for the synthesis of ppGpp dependent on RelA and SpoT. RelA is a ribosome-associated (p)ppGpp synthase, which is activated when an uncharged tRNA binds the ribosome A site as a result of amino acid limitation. RelA then synthesizes pppGpp and ppGpp by phosphorylation of GTP or GDP using ATP as phosphate donor (98, 99). ppGpp was recently shown to bind at an interface of ω and β' subunits of RNA polymerase, thereby acting as an allosteric effector to inhibit global gene transcription, while stimulating the expression of only a few genes related to the synthesis of amino acids (100). rRNA and tRNA synthesis are primarily inhibited, resulting in the global downregulation of bacterial metabolism. SpoT is a bifunctional (p)ppGpp synthase and hydrolase, which presumably regulates the (p)ppGpp level in response to nutrient deficiency. The mechanism by which SpoT senses starvation and synthesizes ppGpp is unclear (101). Many other bacterial species including *Bacillus subtilis* contain only one RelA-SpoT homologue, designated as Rel, which possesses both (p)ppGpp synthase and hydrolase activity. RelA-SpoT homologues have also been recently detected in plants (102). Two *B. subtilis* genes, yjbM and ywaC, were found to encode a novel (p)ppGpp synthase that corresponds to the synthase domain of RelA-SpoT family members while having a different mode of action (103).

Another mechanism by which bacteria regulate gene expression using uncharged tRNA as the effector molecule has been demonstrated in *B. subtilis* and other Gram-positive bacteria. In these organisms the expression of aminoacyl-tRNA synthetase genes and genes involved in amino acid biosynthesis and uptake are regulated by the T box control system [reviewed in (104)]. Regulation by the T box mechanism most commonly

occurs at the level of transcription attenuation (105). The 5′ untranslated regions of regulated genes contain a 200-300 nt conserved sequence and structural element (a G + C-rich helix followed by a run of U residues) that serves as an intrinsic transcriptional terminator and can also participate in formation of an alternate, less stable antiterminator structure. During amino acid starvation binding of a specific uncharged tRNA stabilizes the antiterminator and in doing so prevents formation of the terminator helix. The T box binds specific uncharged tRNA at 2 conserved sites: the anticodon of the tRNA interacts with the codon sequence of the specifier loop (SL) in the 5′-UTR, while the 3′ acceptor end interacts with the UGGN sequence found in the antiterminator bulge, thus stabilizing the structure of the antiterminator and preventing the formation of the competing terminator. RNA polymerase then continues past the terminator region and synthesizes the full length mRNA. Recently a unique mechanism of tRNA-dependent regulation at the transcriptional level was discovered. Saad *et al* found a two-codon T-box riboswitch binding two tRNAs in *Clostridium acetobutylicum*. This T-box regulates the operon for the essential tRNA-dependent transamidation pathway and harbors a SL with two potential overlapping codon positions for $tRNA^{Asn}$ and $tRNA^{Glu}$. Both tRNAs can efficiently bind the SL *in vitro* and *in vivo*. This feature allows the riboswitch to sense two tRNAs and balance the biosynthesis of two amino acids (106). Regulation at the level of translation initiation has also been demonstrated for T box riboswitch in certain bacteria (107). Translationally regulated leader RNAs include an RNA element with the ability to sequester the Shine-Dalgarno (SD) sequence by pairing with a complementary anti-SD (ASD) sequence. Binding of uncharged tRNA stabilizes a

structure analogous to the antiterminator that includes the ASD sequence, and formation of this alternate structure releases the SD sequence for binding of the 30S ribosomal subunit, thereby enabling translation of mRNA coding for proteins involved in amino acid biosynthesis (104).

Uncharged tRNAs also function as regulators in eukaryotes. In amino acid-starved yeast and mammalian cells, uncharged tRNA activates a protein kinase named Gcn2p that phosphorylates eIF2 and thereby reduces the formation of the ternary complex required for translation initiation, while allowing for selected mRNAs, such as GCN4, to be translated. Elevated levels of GCN4, which acts as a transcription factor, stimulates the expression of genes involved in amino acid biosynthesis [reviewed in (108)]. The above mechanisms demonstrate that under certain nutritional stresses, the aminoacylation levels of tRNAs change and the accumulated uncharged tRNAs participate in numerous biological pathways that regulate global gene expression levels, helping the organism to survive under adverse conditions.

Aminoacyl-tRNAs as non-ribosomal substrates

In recent years, the diverse roles of aa-tRNAs have received a great deal of attention. While much of the research has focused on the use of aa-tRNA by the ribosome for protein synthesis, a number of studies have uncovered roles for aa-tRNAs as substrates in other biochemical processes, such as cell wall formation, protein labeling for degradation, aminoacylation of phospholipids in the cell membrane and antibiotic biosynthesis. In this

section we will briefly review some of these various processes that use aa-tRNAs as substrates.

1.3.2 Aminoacyl-tRNA dependent building of peptidoglycan bridges

Peptidoglycans (PG) are structural components of bacterial cell walls that can both serve as a barrier to environmental challenges and provide a scaffold for the attachment of various proteins including virulence factors (109). Peptidoglycan is a polymer of β (1-4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), with all lactyl groups of MurNAc substituted with stem peptides, typically comprised of alternating D- and L-amino acids with an overall common structure of L-Ala-c-D-Glu-X-D-Ala-D-Ala. The composition of the peptide varies among different bacteria: Gram-negative bacteria and Gram-positive bacilli have meso-diaminopimelic acid (DAP) as the third amino acid (DAP-type peptidoglycan), whereas most other Gram-positive bacteria (including Gram-positive cocci) have L-lysine as the third amino acid (110). The stem peptides from adjacent strands are often crosslinked, either directly or through short peptides between the X position of the first pentapeptide side chain with the L-Ala at the fourth position of another. The amino acids required for bridge formation are typically derived from aminoacylated-tRNA donor molecules and are transferred onto the pentapeptide by tRNA-dependent aminoacyl-ligases which catalyze peptide-bond formation by using aminoacyl-tRNAs and peptidoglycan precursors as donor and acceptor, respectively.

The peptidoglycan in *Streptococcus pneumoniae* contains a "stem peptide" composed of up to five amino acids, Ala-γ-D-Glu-Lys-D-Ala-D-Ala, with a L-Ala-L-Ala or a L-Ser-L-Ala dipeptide branch that is attached to the third L-Lys of the pentapeptide side chain. MurM is responsible for the addition of either L-Ala or L-Ser as the first amino acid of the cross-link and then MurN invariably adds L-Ala as the second amino acid (111). In both cases, appropriately aminoacylated-tRNA species serve as the amino acid donors for the reaction (112), although MurM also efficiently accepts mischarged tRNA substrates (113, 114). In *Enterococcus faecalis* BppA1 and BppA2 add L-Ala-L-Ala dipeptide to the pentapeptide chain (115), while FemXAB from *Staphylococcus aureus* sequentially adds one (FemX) or two (FemA and FemB) glycines (116). Lif and Epr in *Staphylococcus simulans* and *Staphylococcus capitis*, FemX in *Weissella virides* and FemX and VanK in *Streptomyces coelicolor* all catalyze similar reactions using aa-tRNAs as substrates [reviewed in (117)].

How aa-tRNAs are diverted from protein synthesis and used as substrates by these enzymes remains somewhat unclear in most instances. In *S. aureus* the mechanism of escape from the protein synthesis machinery could be explained by the observation that three out of the five $tRNA^{Gly}$ isoacceptors encoded in the *S. aureus* genome have sequence identity elements consistent with weak binding to EF–Tu (118). These specific tRNA sequence elements include replacement of the strong EF–Tu binding pairs G49–U65 and G51–C63 [23–25] with A49–U65 and A51–U63, respectively, in the T loop (119, 120). The three non-proteinogenic $tRNA^{Gly}$ isoacceptors also show replacement of GG at positions 18 and 19 with either UU or CU. Hence the issoacceptors with week

binding to EF-Tu could escape protein synthesis and thus allows *S. aureus* to maintain adequate supply of Gly-tRNA^{Gly} for two essential processes: translation and cell wall modification (117).

The specificity of peptidoglycan-modifying enzymes with respect to amino acid and tRNA substrates was demonstrated in the Fem X enzyme from *Weissella. viridescens*. In *W. viridescens* the peptide bridge is made up of L-Ala-L-Ser or L-Ala-L-Ser-L-Ala. FemX initiates peptide bridge formation by transfer of the first L-Ala residue to the amino group of L-Lys found at the third position of the pentapeptide side chain. The enzymes involved in the subsequent transfer of the second position Ser and third position Ala residues have not yet been identified. FemX has a preference for L-Ala addition to UDP-MurNAc pentapeptide because it reacts much more unfavorably with both L-Ser and the acceptor arm of tRNA^{Gly}. *In vitro* assays show that FemX turns over Ser-tRNA^{Ser} and Gly-tRNA^{Gly} 17- and 38-fold less efficiently than Ala-tRNA^{Ala}, respectively. In the latter case, the penultimate base pair of tRNA^{Ala}, G2-C71, was identified as an essential identity element for FemX. This is typically replaced by C2-G71 in tRNA^{Gly} species (121). L-Ala is preferred 110-fold over D-Ala, suggesting relatively weak specificity towards different stereoisomers. The exclusion of serine is due to steric hindrance at the FemX$_{Wv}$ active site rather than poor recognition of the nucleotide sequence of tRNA^{Ser}. Hence, Fem enzymes discriminate non-cognate aa-tRNAs on the basis of both the aminoacyl moiety and the sequence of the tRNA.

1.3.3 Aminoacyl-tRNA dependent aminoacylation of membrane lipids

Bacteria are frequently exposed to cationic antimicrobial peptides (CAMPs), for example eukaryotic host defense peptides or prokaryotic bacteriocins, whose cationic properties impart strong affinities to the negatively charged bacterial lipids phosphatidylglycerol (PG) and cardiolipin (CL). Many bacteria, among them several important human pathogens, achieve CAMP resistance using MprF proteins, a unique group of enzymes that aminoacylate anionic phospholipids with L-lysine or L-alanine, thereby introducing positive charges into the membrane surface and reducing the affinity for CAMPs (122). MprF was first identified when its inactivation rendered a *S. aureus* transposon mutant susceptible to a wide range of cationic antimicrobial peptides (CAMPs) leading to the name 'multiple peptide resistance factor' (MprF) (123). MprFs can use lysyl or alanyl groups derived from aminoacyl tRNAs for modification of PG (124). MprF proteins are integral membrane proteins made up of a C-terminal hydrophilic cytoplasmic domain responsible for the transfer of amino acid onto PG, and an N-terminal transmembrane hydrophobic domain that flips newly synthesized LysPG to the membrane outer leaflet (125). MprF homologues can be found in most bacterial phyla and are abundant in firmicutes, actinobacteria and proteobacteria with the exception of enterobacteria. Some archaea also harbor genes for MprF, probably resulting from lateral gene transfer events (126). MprF homologues exhibit differential specificity for the aa-tRNA substrate they use to modify PG, resulting in a broader classification of these enzymes as aminoacyl-phosphatidylglycerol synthases (aaPGS) (127, 128). For example, the MprFs in *S. aureus* and *P. aeruginosa* only synthesize Lys-PG or Ala-PG, respectively (127,

129). In contrast, *Enterococcus faecium* MprF2 exhibits rather relaxed specificity for the donor substrate and produces both, Ala-PG and Lys-PG, along with small amounts of Arg-PG (130). *L. monocytogenes* MprF is less strict in its specificity for the acceptor substrate and generates both, Lys-PG and Lys-CL (131, 132). Based on the ability of MprF1 to efficiently recognize $tRNA^{Ala}$, $tRNA^{Pro}$, and a $minihelix^{Ala}$ and recognition of the $tRNA^{Lys}$ species from both *Borrelia burgdoferi* and humans, which share less than 50% sequence identity, Roy *et al* proposed that the specificity of MprF arises from direct recognition of the aminoacyl moiety of aa-tRNA (124). The mechanism utilized by MprF and other similar enzymes raises the question of how aa-tRNA donor substrates are directed into membrane lipid modification and away from protein synthesis. Determination of the K_Ds of Lys-tRNA for EF-Tu and for MprF suggested that the two proteins have similar affinities for tRNA under physiological conditions (124). Comparison of the sites in tRNA recognized by MprF and EF-Tu would give a better understanding of how aa-tRNAs are partitioned between translation and membrane lipid modification pathways.

1.3.4 Role of aa-tRNA in antibiotic biogenesis

In addition to having essential roles in protein synthesis and non-ribosomal peptide bond formation, aminoacyl-tRNAs are also used in pathways where the donated amino acid moiety undergoes transformation into a significantly different compound. These pathways involve different amino acid-tRNA pairs and a variety of acceptor molecules (32). Examples of aa-tRNA dependent addition of amino acids in antibiotic biogenesis,

which have been reviewed in detail previously, include valanimycin, pacidamycin and cyclodipeptide synthesis, (117).

Valanimycin, is a potent antitumor and antibacterial azoxy compound first isolated from *Streptomyces viridifaciens* by Yamato and co-workers (133). A gene cluster has been identified that contains 14 genes involved in the biosynthesis of valanimycin (134). The functions of the products of eight of these genes have now been established. Valanimycin is derived from L-Val and L-Ser via an isobutylhydroxylamine intermediate. VlmD, VlmH and VlmR catalyze the conversion of valine into isobutylhydroxylamine, while VlmL catalyzes the formation of L-seryl-tRNA from L-serine. VlmA, which is a homologue of the housekeeping SerRS, catalyzes the transfer of L-serine from L-seryl-tRNA to isobutylhydroxylamine, to produce *O*-(L-seryl)-isobutylhydroxylamine, while VlmJ and VlmK catalyze the phosphorylation and subsequent dehydration of the biosynthetic intermediate valanimycin hydrate to form valanimycin (135). The mechanism by which Ser-tRNA^{Ser} is directed away from translation into the valanimycin pathway, and the identity elements of tRNA^{Ser} that help in recognition by VlmA and VlmL, are still unknown.

Other examples of antibiotics derived from aa-tRNAs are the cyclodipeptides (CDP), a large group of secondary metabolites with a notable range of clinical activities (136-147). It was originally proposed that formation of the CDPs was catalyzed by non-ribosomal peptide synthetases, which do not use aa-tRNAs as substrates. However, subsequent characterization of synthesis of the CDP albonoursin in *Streptomyces noursei* identified the tRNA-dependent CDP synthase AlbC (148). AlbC synthesizes the

albonoursin precursor cyclo (L-Phe-L-Leu) from aminoacylated tRNAs in an ATP-independent reaction (148, 149). CDP synthase products identified to date include cyclo(L-Leu-L-Leu) (cLL), cyclo(L-Phe-L-Leu) (cFL), cyclo(L-Tyr-L-Tyr) (cYY), and cyclo(L-Trp-L-Xaa) (cWX), all of which are intermediates in antibiotic synthesis (150). CDP synthases use their two aa-tRNA substrates in a sequential ping-pong mechanism, with a similar first catalytic step: the binding of the first aa-tRNA and subsequent transfer of its aminoacyl moiety to the conserved serine residue of the enzyme pocket (e.g. Ser37 in the AlbC enzyme) (151). The mechanism of addition of the second amino acid remains unclear, as do the specificity determinants for CDP synthases. Recently, similarities between the predicted secondary structure for PacB, a protein involved in the biosynthesis of the antibiotic pacidamycin, and structures of two Fem transferases led to the characterization of PacB as an ala-tRNA dependent transferase (152). Pacidamycins are a family of uridyl tetra/pentapeptide antibiotics produced by *Streptomyces coeruleorubidus* with antipseudomonal activities through inhibition of the translocase MraY during bacterial cell wall assembly. Analogous to the activity of CDP synthases, PacB hijacks aa-tRNAs and transfers L-Ala from aminoacyl-tRNA donors to the N terminal m-Tyr_2 residue of the growing PacH-anchored antibiotic scaffold (153).

1.3.5 tRNA dependent addition of amino acids to the amino-terminus of proteins

Protein degradation plays an important role in maintaining cellular physiology and in regulation of various cellular processes such as cell growth, differentiation and apoptosis by removing damaged polypeptides and regulatory proteins in a timely manner. As

compared to cellular compartments like lysosomes and vacuoles where proteases are involved in non-specific degradation of proteins, protein degradation in the cytosol of prokaryotes and eukaryotes is often strictly targeted to protect cellular proteins from unwanted degradation. One means to achieve specificity involves the aa-tRNA transferases, which recognize a secondary destabilizing residue (pro-N degrons) at the N-terminus of a target peptide and utilize an aminoacyl-tRNA to transfer a primary destabilizing amino acid (N-degron) to the N-terminal residue, making the protein a target for the cellular degradation machinery (N-recognins) (154). This specificity in protein degradation was discovered in 1986 by Varshavsky and coworkers when they found that different genetic constructs of β-galactosidase proteins from *E. coli* exhibited very different half-lives when produced in *S. cerevisiae*, ranging from more than 20 h to less than 3 min, depending on the identity of their N-terminal amino acid [the N-end rule (155)]. The N-end rule relates the identity of the N-terminal residue of a protein to its *in vivo* half-life (154) and has been shown to function in bacteria (156), fungi (155), plants (157) and mammals (158). In eukaryotes, an N-terminal Arg residue is the preferred N-degron and acts as a target for ubiquitin conjugation and subsequent degradation by the eukaryotic proteasome (159). The degron is generated by the *ATE1* gene product arginyl (R)-transferase, which transfers Arg from Arg-tRNA to the N-terminal α-amino group of oxidized cysteine, Asp, or Glu, which constitute secondary destabilizing residues (Pro-N-degrons) (160, 161). In prokaryotes, Leu and Phe act as the primary destabilizing N-terminal residues (N-degrons) and can be generated by two classes of aa-transferases, leucyl/phenylalanyl(L/F)-transferase encoded by the *Aat* gene and leucyl-transferase

encoded by *Bpt*. The L/F- transferase attaches a primary destabilizing residue of either Leu or Phe to the secondary destabilizing residues Lys, Met and Arg (162), whereas *Bpt*-encoded L-transferase attaches Leu to the secondary destabilizing residues Asp and Glu (161). The Leu/Phe N-degron acts as a target for ClpS, which transfers the protein to ClpAP for subsequent degradation (154). The question that next arises is how the aa-tRNA transferases achieve specificity in binding aa-tRNAs? The crystal structure of leucyl/phenylalanyl-tRNA-protein transferase and its complex with an aminoacyl-tRNA analog solved by Suto *et al.* revealed that the side chain of Leu or Phe is accommodated in a highly hydrophobic pocket, with a shape and size suitable for hydrophobic amino-acid residues lacking a branched β-carbon, such as leucine and phenylalanine (163). The adenosine group of the 3' end of tRNA is recognized largely through π–π stacking with conserved Trp residues. However, L/F transferases achieve specificity for aa-tRNAs through specific interaction with the aminoacyl moiety and not the tRNA, and only the presentation of the specific aminoacyl moiety by a single stranded RNA region is required for recognition (164). The activity of L/F-transferases is reduced in the presence of an excess of EF-Tu, suggesting that L/F-transferase and EF-Tu compete for binding to aa-tRNA.

1.3.6 tRNA-derived fragments

Small non-coding RNA (sncRNA) molecules are major contributors to regulatory networks that control gene expression, and significant attention has been directed towards their identification and studying their biological functions. sncRNA was first discovered

in 1993 in *Caenorhabditis elegans,* and since then a large number of sncRNAs have been identified. sncRNAs are 16–35 nucleotides (nts) long and are classified into different groups such as microRNA (miRNA), small-interfering RNA (siRNA), piwi-interacting RNA and small nucleolar RNA (snoRNA). Among them, miRNA and siRNA are the most extensively studied, and both suppress gene expression by binding to target mRNAs. The recent development of high-throughput sequencing technology has improved the identification of other types of small RNAs like tRNA-derived RNA fragments (tRFs) which have been identified by several research groups (165). There is increasing evidence that these are not by-products from random degradation, but rather functional molecules that can regulate translation and gene expression. The production of tRNA fragments and their emerging roles in the cell are discussed below.

Production of tRNA fragments

tRNA halves

tRNA halves are composed of 30-35 nucleotides derived from either the 5′ or 3′ part of full-length mature tRNA. These tRNA halves are produced by cleavage in the anticodon loop under nutritional, biological, physicochemical or oxidative stress (166-168). In mammalian cells tRNA halves are generated during stress conditions by the action of the nuclease angiogenin, a member of the RNase A family (169) whereas in yeast Rny1p, a member of the RNase T2 is responsible for tRNA half production. Apart from their roles as nucleases, both angiogenin and Rny1 act as sensors of cellular damage and can promote cell death and inhibit tumor formation (96, 170). Under normal conditions both

ribonucleases are either sequestered in the nucleus or, in the case of angiogenin, bound to its inhibitor RNH1, and are released into the cytoplasm under certain stress conditions (171). tRNA halves have also been identified in bacteria, archaea and plants . In bacteria tRNA anticodon nucleases like PrrC, colicin D, and colicin E5 have been shown to cleave specific subsets of tRNAs [reviewed in (172, 173)] (Fig. 1.4).

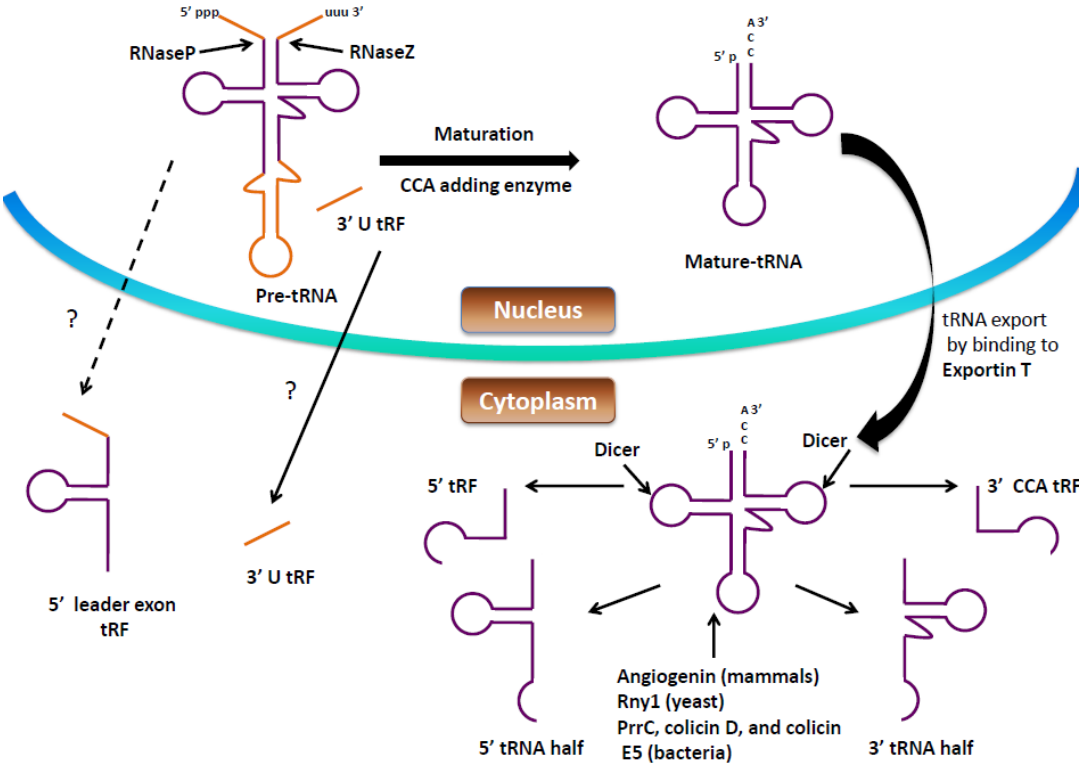


Figure 1.4. Formation of small RNAs from tRNA. Precursor tRNA are processed by RNase P, RNase Z, the splicing endonuclease and CCA-adding enzyme to form mature tRNA in the nucleus. Processing of both the pre-tRNA and mature tRNA can give rise to small RNA. The figure shows possible routes for small RNA (tRNA halves, 5' tRF, 3' CCA tRF, 3' U tRF and 5' leader exon tRF) production from tRNA. The dashed lines and question marks indicate mechanisms of formation or transport of these tRFs that are not clear.

tRNA fragments

tRNA-derived fragments (tRFs) are shorter than tRNA halves, ranging between 13–20 nts in size. They have been identified in all domains of life. There are four types of tRFs known and they are classified based on the part of the mature tRNA or pre-tRNA from which they are derived. tRFs were classified as 5′ tRFs, 3′ CCA tRFs, 3′ U tRFs, or 5′

leader-exon tRFs. 5’ tRFs are derived from the 5’ end of the tRNA generated at any point of tRNA processing, provided the 5’ leader sequence is removed by RNaseP, and are formed by a cleavage in the D loop. In the case of 5' tRFs their biogenesis is carried out by Dicer in mammalian cells (174). However, it is known that the Dicer-independent generation of 5' tRFs takes place in *Schizosaccharomyces pombe* due to the differences in length of the 5’ tRFs generated in these two organisms (19 nt long in mammals and 23 nt long in yeast), suggesting that a protein other than Dicer is responsible for their production in yeast (175). 3′ CCA tRFs are produced from the 3’ ends of mature tRNA by cleavage at the T loop and carry the trinucleotide CCA at the acceptor stem. Dicer has been implicated in the generation of the 3’ end fragment (176), although angiogenin and other RNase A members have also been proposed to function in Dicer-independent processing (96, 177). 3′ U tRFs are cleaved from the 3’ end of tRNA precursors by RNase Z, and their biogenesis is normally Dicer independent. They commonly start directly after the 3’ end of mature tRNAs and end in a stretch of U residues produced by RNA polymerase II run-off (165, 178). One 3’ U tRF is produced in an RNaseZ independent manner by the action of Dicer on the predicted bulged hairpin structure of the pre-tRNA (179). The mechanism of formation of 5’ leader-exon tRFs are not known, however they have been identified in CLP1 mutant cells possibly arising due to aberrant splicing. CLP1 is an RNA kinase and is a component of the mRNA 3’ end cleavage and polyadenylation machinery in mammals (180).

While it was previously thought that production of tRNA halves and tRFs were solely mechanisms to remove damaged tRNAs, increasing evidence suggests their

formation to be regulated. Angiogenin and Rny1 involved in the production of tRNA halves are usually sequestered in compartments before they are released in the cytoplasm where they cleave tRNAs (181). However the regulation of their release from these cellular compartments is not known. Also a number of tRNAs (including $tRNA^{Asp(GTC)}$, $tRNA^{Val(AAC)}$ and $tRNA^{Gly(GCC)}$) can be methylated by Dnmt2, which has been shown to protect these tRNAs from cleavage during stress (182). This specificity in cleavage of tRNAs might be responsible for the different types of tRFs observed under various conditions.

Functions of tRFs

Are tRFs merely the products of tRNA degradation or do they have *bona fide* biological functions? If so, how diverse are these functions given the various forms of tRFs identified? Several lines of evidence point toward regulated production, suggesting that they may be functional RNA species. First, the abundance of different types of tRF does not correlate with the number of parent tRNA gene copies (174, 183-185), with the exception of those found in *Tetrahymena* (186). Second, the fragments of tRNA formed are produced by cleavage at specific points in the tRNA. Third, whilst tRFs corresponding to the 5' and 3' ends of tRNA have been reported, those corresponding to the middle (incorporating the anticodon loop) have not. Although, the exact roles of tRNA halves and tRFs are yet to be elucidated, accumulating evidence suggests that tRNA-derived small RNAs participate in two main types of biological processes as discussed in more detail below.

Translation regulation of gene expression under stress conditions

tRNAs are indispensible components of the translational machinery, hence tRNA cleavage under stress conditions can affect protein synthesis. However the mode of translational regulation by tRNA cleavage is not that simple. It has been shown previously that during stress conditions, formation of tRNA cleavage products does not change the pool of full length tRNA significantly, rather these fragments represent only a small portion of the tRNA pool (171). Ivanov *et al* showed a more intricate role for tRNA halves in translational control (187). They observed that tRNA halves formed by angiogenin during stress were able to inhibit protein synthesis and trigger the phospho-eIF2α-independent assembly of stress granules (SGs). These granules are mainly composed of stalled pre-initiation complexes, suggesting that the translational initiation machinery can be targeted by 5′ tRNA halves. They demonstrated that selected tRNA halves inhibit protein synthesis by displacing eIF4G/eIF4A from capped and uncapped mRNA and eIF4E/G/A (eIF4F) from the m^7G cap. Using pull down of 5′-tiRNA^{Ala} – protein complexes the authors implicated YB-1, a translational repressor known to displace eIF4G from RNA and eIF4E/G/A from the m^7G cap (188, 189). Analysis of the 5' tRNA halves in complex with YB-1 revealed that a terminal oligo-G motif containing four to five consecutive guanosines present in certain 5′ tRNA halves (Ala/Cys) was absolutely required for translational repression of a reporter mRNA, suggesting the inhibition is caused by specific tRNA and is not a consequence of global upregulation of tRFs (187). This result came as a surprise as regulation of translation during stress is

carried out phosphorylation of eIF2 (Section II), which induces translational repression facilitated by active sequestration of untranslated mRNAs into SGs (190).

In addition to tRNA halves, tRFs have also been implicated in regulation of translation. In the archaeon *Haloferax volcanii* a 26 nt-long 5'tRF originating from $tRNA^{Val}$ in a stress-dependent manner was shown to directly bind to the small ribosomal subunit and inhibit translation by interfering with peptidyl transferase activity (191). A similar mechanism of translation inhibition by a 5'tRF was recently observed in human cells (192). A 26 nt 5' tRF derived from $tRNA^{Val}$ was able to inhibit translation by affecting peptide bond formation. An interesting observation from this study was that the tRFs required a conserved "GG" dinucleotide for their activity in inhibiting translation. A similar motif dependence is observed as discussed above in translation inhibition by a 5' tRNA half. 5' tRNA halves containing the 5' tRF sequence were shown to require a run of at least four guanosine residues at the 5' end of the molecule, which is present only in $tRNA^{Ala}$ and $tRNA^{Cys}$, as compared to 5' tRFs that require only two guanosine residues at the 3' end of the molecule, residues conserved between tRNAs. Mutating the di-guanosine motif required by 5'tRF in the 5' tRNA half did not affect its inhibitory activity, and the precise mechanism of translation inhibition by these tRFs warrants further investigation (192).

tRNA-derived fragments as regulators of gene silencing

One of the first studies showing the involvement of tRNA-derived fragments in gene regulation and silencing was carried out by Yeung *et al.* who addressed the role of small

RNAs in human immunodeficiency virus (HIV) infected cells. A highly abundant, 18 nt long, tRF originating from the 3' end of human cytoplasmic $tRNA^{Lys3}$ was shown to target the the HIV-1 primer-binding site (PBS) similarly to siRNAs that target complementary RNA (193). $tRNA^{lys}$ is used by viral reverse transcriptases as primer for the initiation of reverse transcription and DNA synthesis (194). The 3' tRF was shown to be associated with Dicer and AGO2, and to cause RNA cleavage of the complementary PBS sequence thereby showing the role of a tRF in viral gene silencing. Other tRFs like 3'CCA, 5' and 5' U tRF have also been shown to be associated with agronautes and hence have a potential to function as an siRNA or miRNA. Haussecker *et al*. investigated the ability of 3' CCA and 3'U tRFs to associate with Argonaute proteins and cause silencing of a reporter luciferase transgene (178). They found that both types of 3' tRF associated with Argonaute proteins, but often more effectively with the nonsilencing Ago3 and Ago4 than Ago1 or Ago2. They observed that 3' CCA tRFs had a moderate effect on reporter transgene silencing, but 3'U tRFs did not. However, upon co-transfection of a small RNA complementary to the 3' U tRF, the tRF preferentially associated with Ago2 and caused 80 % silencing of the reporter transgene. This correlated with redirection of the reconstituted fully duplexed double-stranded RNA into Ago 2, whereas Ago 3 and 4 were skewed toward less structured small RNAs, particularly single-strand RNAs. This is in stark contrast with results normally obtained in the miRNA field where sequences complementary to miRNAs relieve repression, a phenomenon known as sense-induced transgene silencing (SITS). Modulation of tRF levels had minor effects on the abundance of microRNAs, but more pronounced changes

in the silencing activities of both microRNAs and siRNAs. This study provides compelling evidence that tRFs play a role in the global control of small RNA silencing through associating with different Argonaute proteins (178).

A tRF that functions as an miRNA was recently described, a 22 nt 3'tRF generated in a Dicer-dependent manner from $tRNA^{GLy}$ in mature B cells and associated with Argonaute proteins (176). The 3'tRF was shown to inhibit RPA1, an essential gene involved in DNA repair by possibly binding to the 3'UTR region. Expression of this 3' tRF was down regulated in a lymphoma cell line indicating that loss of 3' tRF expression might help the cancer cells to tolerate the accumulation of mutations and genomic aberrations during tumor progression.

Other Biological functions of tRFs

Apart from the two known biological functions of tRFs in regulation, other potential biological functions are beginning to be identified. Recently a study by Ruggero *et al.* showed their role in viral infectivity. Large scale sequencing of small RNA libraries was used to identify small noncoding RNAs expressed in normal $CD4^+$ T cells compared to cells transformed with human T-cell leukemia virus type 1 (HTLV-1), the causative agent of adult T-cell leukemia/lymphoma. Among the miRNAs and tRFs expressed, one of the most abundant tRFs found was derived from the 3' end of $tRNA^{Pro}$, and exhibited perfect sequence complementarity to the primer binding site of HTLV-1. *In vitro* reverse transcriptase assays verified that this tRF was capable of priming HTLV-1 reverse transcriptase thereby suggesting an important role in viral infection. One possible role

suggested for the tRF fragment is to support the initiation of reverse transcription, but not progressivity, with failure to proceed to the strand transfer step (195). Further studies are now needed to compare the abilities of the tRF and of full length $tRNA^{Pro}$ to prime and support strand transfer. Variation of tRNA halves accumulation was also shown in the parasites Toxoplasma *gondii*, the agent of toxoplasmosis, and the rodent malaria parasite *Plasmodium berghei*. These organisms exhibited increased tRNA accumulation upon egress from host cells and in response to stage differentiation, amino acid starvation and heat-shock. It was observed that avirulent isolates of *T. gondii* and attenuated *P. berghei* parasites displayed higher rates of tRNA cleavage compared to virulent strains. Also tRNA half production was significantly higher in the metabolically quiescent bradyzoite and sporozoite stages of *T. gondii*, compared to the fast-growing tachyzoite indicating a relationship between half-tRNA production and growth rate in this important group of organisms (196). A role for tRF halves in Respiratory Syncytial Virus (RSV) infectivity was recently shown by Wang *et al.* who observed an induction of tRNA cleavage upon RSV infection with a specific subset of tRNAs being cleaved. The 31 nt 5'tRF(Glu) formed exhibited *trans*-silencing capability against target genes, however the mechanism of gene silencing was found to be different than the gene-silencing mechanism of miRNA/siRNA, previously also shown for other tRFs. Interestingly the tRF was also shown to promote RSV replication (197)

tRNA fragments have also been implicated in progressive motor neuron loss. Hanada *et al.* recently demonstrated that tRNA fragments generated in CLP1 mutant cells sensitize cells to oxidative stress-induced activation of the p53 tumor suppressor pathway and in

turn lead to progressive loss of spinal motor neurons leading to muscle denervation and paralysis thereby providing a possible link between tRNA cleavage and p53 dependent cell death. However, the exact mechanism by which these tRNA fragments affect the p53 pathway needs to be determined (180).

1.3.7 Regulation of cell death by tRNA

Apoptosis is a cellular process by which damaged, harmful and unwanted cells are eliminated. Apoptotic regulation is critical to cell homeostasis, immunity, multi-cellular development and protection against infections and diseases like cancer (198). Apoptotic cells have been shown to undergo various morphological and biochemical changes caused by a group of **c**ysteine-dependent **a**spartate **s**pecific **p**rote**ases,** or caspases. In healthy cells caspases are inactive, however during apoptosis caspases are activated and signal the onset of apoptosis via cleavage of various intracellular proteins including apoptotic proteins, cellular structural and survival proteins, transcriptional factors, signaling molecules and proteins involved in DNA and RNA metabolism (199, 200). Cleavage of these intracellular proteins ultimately leads to phagocytic recognition and engulfment of the dying cell. While many factors have been discovered that regulate the apoptotic pathway, in this section the recently discovered role of tRNA as a regulator of cell death is discussed.

Caspase activation by extrinsic and intrinsic pathways

Apoptosis can be triggered via two major routes: an extrinsic, or extracellularly-activated pathway and/or an intrinsic, or mitochondrial-mediated pathway. Both pathways activate caspases, a class of endoproteases that hydrolyze peptide bonds (201). Although there are various types of caspases, those involved in apoptosis can be classified into two groups, the initiator (or apical) caspases and the effector (or executioner) caspases. Initiator caspases (e.g. Caspase-8 and 9) are capable of autocatalytic activation, whereas effector caspases (e.g. Caspase-3, 6 and 7) are activated by initiator caspase cleavage (202, 203). The extrinsic pathway begins outside the cell through activation of a group of pro-apoptotic cell surface receptors, such as Fas/CD95 and tumour necrosis factor receptor. Upon binding to their cognate ligand, these receptors recruit an adaptor protein Fas-associated death domain that binds and dimerizes the initiator procaspase-8, to form an oligomeric death-inducing signaling complex, in which procaspase-8 becomes activated through an autoproteolytic cleavage event. The active caspase-8 then cleaves and activates the effector caspases 3 and 7 (199, 204, 205). The intrinsic pathway causes mitochondrial outer membrane permeabilization (MOMP), which leads to release of cytochrome *c*, a mitochondrial inner membrane protein which transfers electrons from complex III to complex IV in the electron transport (206). The discovery of the role of cytochrome *c* in apoptosis by Liu *et al* (207) came as a surprise due to its essential role in the survival of the cell. In the cytosol, cytochrome *c* interacts with the apoptotic protease activating factor-1 (APAF-1) to form the apoptosome complex (208). The complex recruits procaspase-9, which converts to active caspase-9 by autocatalysis. Active

caspase-9 activates effector caspases like caspase-7 and caspase-3 and causes apoptosis (Fig. 1.5). Apoptosis is regulated by several pro-apoptotic proteins (Bax, Bak and Bid), anti-apoptotic proteins (Bcl-2, Bcl-X_L and Mcl-1) and a range of cellular factors (HSP90, HSP70 and HSP27) (209, 210) that is now known to include tRNA.

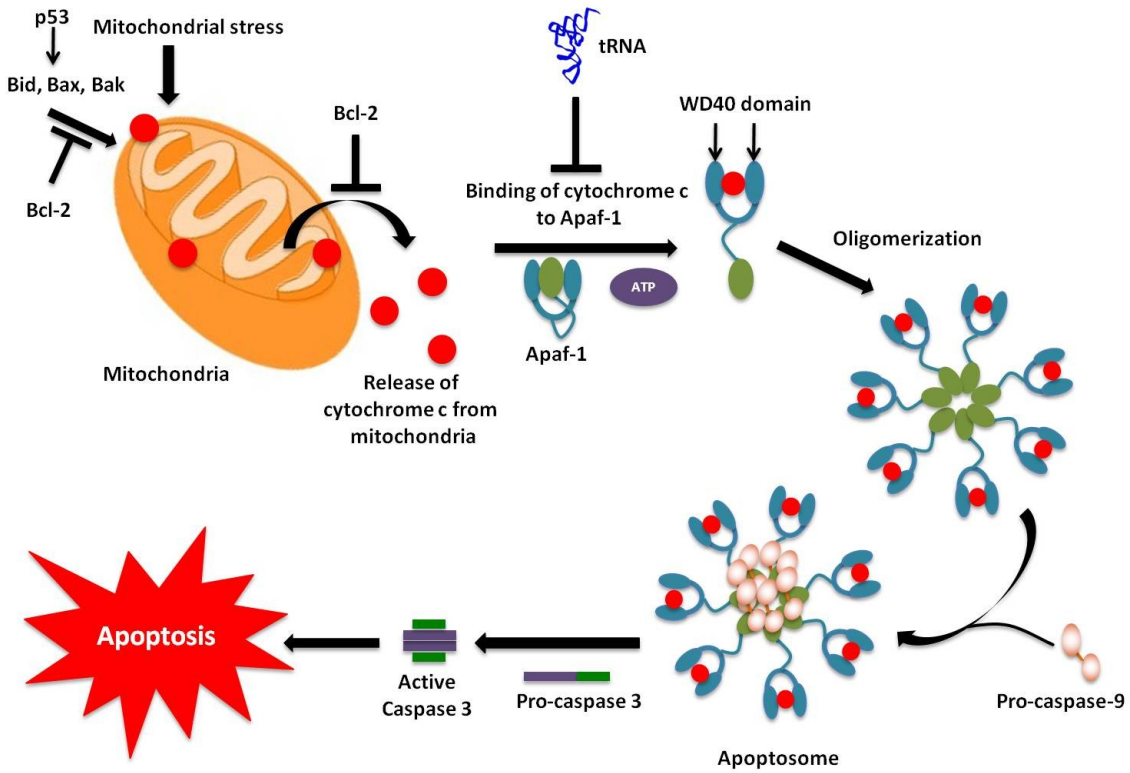


Figure 1.5. Intrinsic pathway for apoptosis. The intrinsic pathway, typically initiated by DNA damage activates p53. p53 then activates the pro-apoptotic proteins, which cause mitochondrial outer membrane permeabilization (MOMP) leading to release of cytochrome *c* into the cytoplasm. In the cytoplasm, cytochrome *c* associates with Apaf-1 to form the apoptosome complex. However, tRNA may interact with cytochrome *c* and prevent its binding to Apaf-1. The apoptosome causes the conversion of inactive pro-caspase-9 into active caspase-9. Caspase-9 then activates caspase-3 that then leads to the caspase cascade, resulting in apoptosis.

Interaction between tRNA and cytochrome *c*: potential role in regulating apoptosis

To answer the long standing conundrum of why 1 mM dATP is required to induce caspase-9 activation in cell lysates, when the intracellular concentration of dATP is only 10 μM, Mei *et al* investigated the role of RNA, which is essentially a polymer of nucleoside monophosphates, in cytochrome *c*-mediated caspase activation. They observed that treatment of mammalian S100 extracts with RNase strongly increased cytochrome *c*-induced caspase-9 activation, while the addition of RNA to the extracts impaired caspase-9 activation. These results implicated an inhibitory role of RNA in the activation of caspase-9. Systematic evaluation of the steps leading to caspase-9 activation identified cytochrome *c* as the target of the RNA inhibitor. Analysis of cytochrome *c* associated species revealed that tRNA binds specifically to cytochrome *c*. Microinjection of tRNA into living cells inhibited the ability of cytochrome *c* to induce apoptosis, while degradation of tRNA by an RNase that preferentially degrades tRNA, onconase, enhanced apoptosis via the intrinsic pathway. Taken together, these findings showed that tRNA binds to cytochrome *c* and inhibits formation of the apoptosome (211). This suggested a direct role for tRNA in regulating apoptosis and revealed an intimate connection between translation and cell death. This finding also raised an interesting question as to how the interaction between tRNA and cytochrome *c* modulates apoptosis. This question was addressed recently by Gorla *et al.* who proposed that interaction of tRNA with the heme moiety of cytochrome c protects the positively charged surface of cytochrome *c* from being exposed to the APAF-1 complex. This model was further confirmed by the observation that cytochrome *c* lost its ability to interact with tRNA after

treatment with oxidizing agents or cysteine modifying agents. In such a state, hemin is unable to bind to tRNA and the exposed positively charged residues of cytochrome *c* then bind to APAF-1 (209). Hence tRNA can regulate apoptosis by binding to cytochrome *c*. Further investigation of the nucleotide residues of tRNA involved in these interactions is required to answer questions about how tRNA binding to cytochrome *c* is regulated in the cell, whether specific tRNA isoacceptors are involved, and if this interaction is nonspecific. Increased expression of tRNA has been detected in a wide variety of transformed cells (212), such as ovarian and cervical cancer (213, 214), carcinomas and multiple myeloma cell lines (215). Expression levels of tRNA molecules in breast cancer cells was 10-fold higher as compared to in normal cells and overexpression of $tRNA_i^{Met}$ induces proliferation and immortalization of fibroblasts and also significantly alteres the global tRNA expression profile (216). It was also observed that certain individual tRNAs were overexpressed more as compared to others. $tRNA^{Arg(UCU)}$, $tRNA^{Arg(CCU)}$, $tRNA^{Thr\ (CGU)}$, $tRNA^{Ser(CGA)}$, and $tRNA^{Tyr(GTA)}$ were among the most over-expressed tRNAs, while $tRNA^{His(GTG)}$, $tRNA^{Phe\ (GAA)}$, and $tRNA^{Met(CAT)}$ were the least over-expressed tRNAs (217) indicating overexpression is not random and may be related to regulation of cytochrome *c*. Identification of the tRNA sites involved in binding to cytochrome *c* might help elucidate the connection between tRNA overexpression and cancer. tRNA cleavage has also been suggested as a mode of regulation of this interaction (199).

1.4 Purpose of study

While the canonical functions of aaRSs are well studied, the non canonical functions like editing and MSC formation are not fully understood. The mammalian MSC has been shown to be involved in the regulation of transcription, translation silencing and various signaling pathways that modulate inflammation, angiogenesis, and autoimmune responses. However the function of this complex in translation remains unclear. Although, MSCs have been suggested to improve the efficiency of translation by channeling of charged tRNA, the direct role of these complexes in translation remains elusive. MSC function investigated in the archaeon *T. kodakarensis*, by characterizing the effect of MSC formation on individual aaRSs activities and studying the possible association of the MSC with the components of the translation machinery. This study will help shed light on how such complexes control the efficiency and accuracy of translation and provide insight into the role of the mammalian MSC.

Also, it is becoming increasingly clear that aaRS have evolved different quality control mechanisms in different organisms. For example, yeast cytoplasmic PheRS has evolved to display low specificity for its cognate amino acid and employs post-transfer editing to clear mischarged Tyr, while mitochondrial PheRS displays high specificity towards Tyr. In contrast, *E. coli* PheRS has evolved both high active site specificity and post-transfer editing to clear mischarged Tyr. Intriguingly, these discrimination mechanisms were found to be dispensable under normal growth conditions. These observations raise questions about the selection pressures that govern evolution of these editing mechanisms. In addition to the well-documented ability of aaRSs to edit tRNAs charged

with genetically-encoded near cognate amino acids, these same proofreading activities have been demonstrated to act on other non-canonical substrates. Both *E. coli* and *Thermus thermophilus* PheRS have also been shown to edit *m*-Tyr, a metabolic byproduct formed by oxidation of phenylalanine. Hence dissecting the different PheRS quality control mechanisms with respect to editing of Tyr isomers in both *E. coli* and yeast will help understand the evolution of these quality control mechanisms.

PheRS and TyrRS activate amino acids that differ by a single hydroxyl group. However, while PheRSs have evolved editing mechanism to clear mischarged Tyr, TyrRSs display very high specificity towards Phe. Despite this high specificity, significant incorporation of Phe residues at Tyr codons was observed during recombinant antibody production. Mis-incorporations have recently been observed under a variety of conditions including oxidative stress, change in amino acid pools, change in codon bias and genetic heterogeneity. The cause and biochemical mechanism of misincorporation by eukaryotic TyrRS will hence be studied to help understand conditions that affect TyrRS quality control.

Chapter 2:

Association of a multi-synthetase complex with translating ribosomes in the archaeon *Thermococcus kodakarensis*

2.1 Introduction

Aminoacyl-tRNA synthetases (aaRSs) attach amino acids (aa) to their cognate tRNAs to form aa-tRNA, which is then delivered to the ribosome for protein synthesis as a ternary complex with translation elongation factor 1A (EF1A) and GTP (218). AaRSs have been found in a variety of complexes with each other and with other factors, potentially expanding the functions of aaRSs both within and beyond translation (219). In mammalian cells 9 aaRSs (ArgRS, AspRS, GlnRS, GluRS, IleRS, LeuRS, LysRS, MetRS and ProRS) associate with three non-synthetase protein factors (p18, p38, and p43) to form a large multi-aminoacyl-tRNA synthetase complex (MSC). The accessory proteins are important for the formation and stability of the complex, promote binding of tRNAs to the complex, and also play other roles outside translation (220-223). In

addition to the MSC, another aaRS, ValRS forms a complex with the human multi-subunit translation elongation factor 1H (EF1H), which increases the catalytic efficiency of $tRNA^{Val}$ aminoacylation (224). In lower eukaryotes, including *S. cerevisiae*, complexes have been characterized between GluRS, MetRS and Arc1p, and between SerRS and the peroxisome biosynthesis factor Pex21p, both of which enhance tRNA binding to their respective aaRSs (225-227).

Although many of these associations were first described in eukaryotic cells, numerous multi-enzyme complexes containing aaRSs have also been identified in both Bacteria and Archaea. In bacteria, complexes comprised of one aaRS and a second non-aaRS protein have been implicated in cellular functions including editing of misacylated tRNAs, indirect synthesis of aa-tRNA and metabolite biosynthesis (228-230). In Archaea, aaRS-containing complexes were first described in *Haloarcula marismortui*, with many aaRSs purified in one or possibly two large complexes, and in *Methanocaldococcus jannaschi* where ProRS was found to interact with a components of the methanogenesis machinery (77, 231, 232). In another archaeal methanogen, *Methanothermobacter thermautotrophicus*, one complex composed of LeuRS, LysRS, ProRS and EF1A was identified while another contained SerRS and ArgRS. In both cases the formation of MSCs was found to improve the catalytic efficiency of tRNA aminoacylation by the aaRSs present in the corresponding complexes (79-83).

Previous studies on aaRS subcellular localization led to the proposal that MSCs directly channel aa-tRNAs to EF1A without dissociation in the cytoplasm (79, 233). This channeling could potentially provide a sequestered pool of aa-tRNAs specifically for

utilization in protein synthesis, although a direct interaction between the ribosome and aaRSs in the MSC has not been demonstrated. The mammalian MSC (MARS) has been shown to interact with polysomes, but whether this reflects substrate channelling during protein synthesis is unclear given the presence of three essential aaRS-interacting factors that also function outside the complex (223, 234). MSCs have been identified in Archaea that do not require aaRS-interacting factors for assembly and function, potentially providing suitable systems to investigate MSC interactions with other components of the translation machinery. Previous attempts to characterize, purify and reconstitute archaeal MSCs have met with some limited success, in part due to the comparative instability of the complex (80). Here a more systematic investigation of an archaeal aaRS interactome is described allowing the characterization of a polysome-associated MSC in the archaeon *T. kodakarensis*, providing evidence for the interaction of aaRSs with the mRNA translation machinery and is consistent with substrate channeling during protein synthesis.

2.2 Materials and methods

2.2.1 Strain construction and protein purification

Construction of shuttle vector pHis$_6$-HA LeuRS and its use to transform *T. kodakarensis* strain KW128 were performed as previously described (235, 236). Plasmids for the production of N-terminally tagged intein fusion derivatives of LeuRS, ProRS and EF1A (TK1461, TK0550 and TK 0308, respectively) were constructed by inserting the

corresponding PCR amplified genes into pTYB11 vector between SapI and XhoI. For the intein tagged LeuRS, ProRS and EF1A constructs the forward primers used were 5 'GTTGTTGTACAGAACATGGCTGAGCTTAACTTTAAG 3', 5' GTTGTTGTACA GAACATGGCGGTTGAGAGAAAGAA 3' and 5' GTTGTTGTACAGAACATGGCT AAGGAGAAG 3' and the reverse primers were 5 'CTGCAGTCACCCGGGTCACTCC ACAAACACCGCGGG 3', 5' CTGCAGTCACCCGGGTCAGTAAGTCCTCGC 3' and 5' CTGCAGTCACCCGGGTCAGTCGGCCTTCTG 3' respectively. Cloning of N terminally His_6 tagged TyRS (TK0568) into pQE31 was done by isolating the gene from the *T. kodakarensis* DNA and ligating into the BamHI and HindIII digested pQE31 plasmid. The primers used were 5' CATACGGATCCGATGGACATAGAAAGGAAG3' and 5' ATTAAGCTTTTACCGGGTTATCTTTATCTC 3' flanked by BamHI and HindIII sites.

The plasmid to express the gene encoding the LeuRS-derived bait for interactome analysis was constructed as follows. The gene encoding LeuRS (*leuS*) was PCR amplified from *T. kodakarensis* DNA and the coding sequence was extended in frame by ligation of an oligonucleotide sequence that added the constitutive promoter from the *M. thermoautotrophicum* archaeal histone-encoding hmtB gene, a mutant RBS (AAGTGG), and also encoded a hemagglutinin (HA) epitope and six histidine residues (His_6) at the N terminus of LeuRS (Fig. 2.1). This gene was then ligated into plasmid pTS414 (28) between SalI and NotI sites. Plasmid MR3 was similar to MR4 except that the RBS site was WT(AGGTGG). The mutant RBS was used to modulate synthesis of tagged LeuRS in the cell (Fig. 2.2).

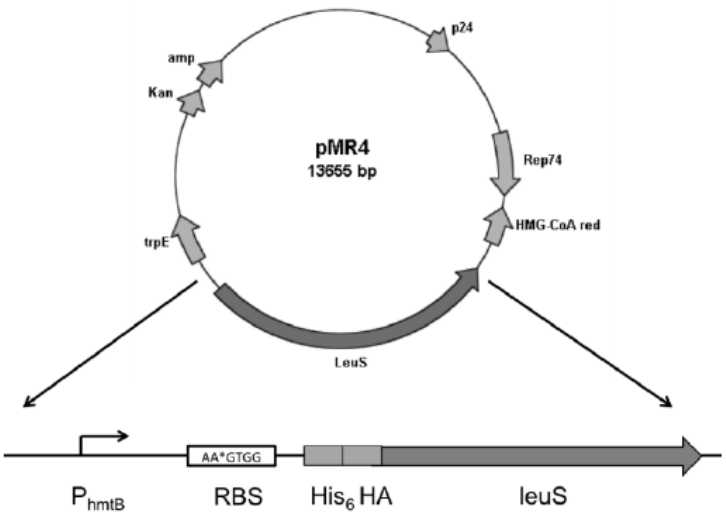


Figure 2.1. Map of plasmid pMR4

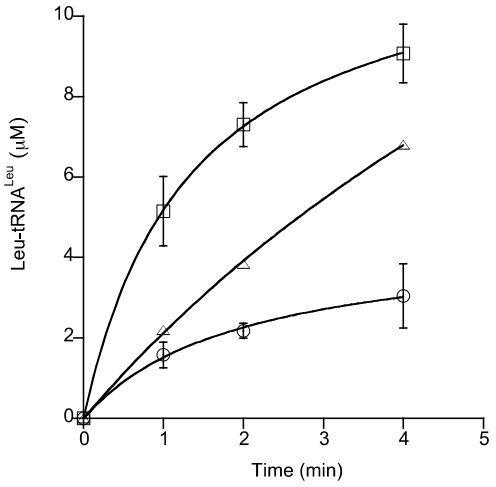


Figure 2.2. Production of LeuRS in *T. kodakarensis* assessed by the aminoacylation activity of LeuRS. Aminoacylation activities of cell free extracts of *T. kodakarensis* KW128 strain transformed with pTS414 plasmid lacking the LeuRS gene(○), pMR3 containing the LeuRS gene and WT RBS (□) and pMR4 (Δ) were measured with 5 μM $tRNA^{Leu}$ after dialyzing against buffer (25 Mm Tris-HCl pH 7.5, 100 mM KCl and 10 mM beta-mercaptoethanol).

Intein tagged LeuRS, ProRS and EF1A were produced by transforming *E. coli* BL21(DE3)pLysS (Stratagene) with pTYB11 vectors containing the respective genes. Protein was produced by first growing a starter culture at 37 °C at 240 rpm until mid log phase was reached and then using this to inoculate a larger culture (1L). The larger culture was grown to OD_{600} of 0.2 at 37 °C and then transferred to 18 °C at 190 rpm for 90 min. Protein expression was induced with 0.5 mM IPTG for 12 h. Cell-free extract was produced by sonication of cells in buffer A [20 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10 % glycerol] containing a protease inhibitor mixture tablet (Complete Mini, EDTA-free; Roche Applied Science) followed by centrifugation at 150,000xg for 45 min. The resulting supernatant was loaded onto a chitin column, washed extensively with buffer A, and cleavage of the intein tag was induced by incubation of the protein on the chitin column with buffer A containing 50 mM dithiothreitol (DTT) for 24 h (all of the following procedures were performed at 4 °C unless otherwise stated). The protein was then eluted with buffer A containing 50 mM DTT. Fractions containing the respective proteins were pooled (judged by Coomassie Brilliant Blue staining after SDS-PAGE) and buffer was exchanged into buffer B (25 mM Tris-HCl, pH 7, 50 mM KCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% glycerol) before loading on to a Resource Q column, or in the case of EF1A a Resource S column (GE Healthcare), to which a KCl gradient (0–500 mM) in buffer B was applied. Eluted fractions containing the desired protein were pooled and dialyzed against storage buffer [25 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 50 % glycerol; for EF1A 10 µM GDP was

also included] prior to storage at -80 °C. His_6-TyrRS was purified as previously described and stored in the above mentioned storage buffer at -80 °C (79).

2.2.2 Isolation and identification of His_6-HA-LeuRS associated proteins from *T. kodakaraensis*

T. kodakarensis KW128 (MR4)/pHis_6-HA-LeuRS (236) was grown to mid exponential phase (OD_{600} = 0.4) at 85 °C in MA-YT medium supplemented with 5 g sodium pyruvate/L and 5 μM mevinolin. Cells were harvested by centrifugation, resuspended in 20 ml of buffer A with 10 mM imidazole and lysed by sonication. After centrifugation, the resulting clarified lysate was loaded onto a 1ml HiTrap chelating column (GE Healthcare) pre-equilibrated with $NiSO_4$. The column was washed with buffer A and proteins were eluted using a linear imidazole gradient from buffer A to 100 % buffer C (25 mM Tris-HCl [pH 8], 100 mM NaCl, 500 mM imidazole and 10 % glycerol). Fractions containing His_6-HA-LeuRS were then identified by carrying out western blot analyses using anti-HA antibodies, and 30 μg aliquots of the positive fractions subsequently precipitated by adding trichloroacetic acid (TCA; 15 % final concentration). TCA-precipitated proteins were identified by multidimensional protein identification technology (MuDPIT) at the Ohio State University mass spectrometry facility (http://www.ccic.ohio-state.edu/MS/ proteomics.htm) using the MASCOT search engine. MASCOT scores >100, indicative of a minimum of two unique peptide fragments identified from the same protein, were considered significant. Protein isolation and mass spectrometry analyses were also performed with lysates of two independent cultures of

wild-type *T. kodakarensis* KW128 to identify untagged proteins that non-specifically bound and eluted from the Ni^{2+}-charged matrix. All proteins identified in the experimental samples that had MASCOT scores >100, but were absent from the control samples, are listed in Table 2.2.

2.2.3 Aminoacylation Assays

T. kodakarensis total tRNA was prepared as previously described (237). The gene encoding *T. kodakarensis* $tRNA^{Leu}$ (GAG anticodon) cloned into pUC19 was used for synthesis and purification of the corresponding *in vitro* transcribed tRNA using standard procedures (238). L-[U-^{14}C] leucine (324 mCi/mmol), L-[U-^{14}C] serine (163 mCi/mmol), L-[U-^{14}C] arginine (346 mCi/mmol), L-[U-^{14}C] phenylalanine (487 mCi/mmol), L-[U-^{14}C] glutamic acid (260 mCi/mmol) and L-[U-^{14}C] tyrosine (482mCi/mmol) were from PerkinElmer Life Sciences. L-[U-^{14}C] proline (269 mCi/mmol), L-[U-^{14}C] aspartic acid (207 mCi/mmol), L-[U-^{14}C] alanine (164 mCi/mmol) and L-[U-^{14}C] lysine (309 mCi/mmol) were from Amersham Biosciences. A reaction mixture containing 100 mM HEPES (pH 7.5), 250 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, bovine serum albumin (200 µg/ml), *T. kodakarensis* total tRNA or *in vitro* transcribed tRNA, and aaRSs at concentrations indicated for specific experiments was first prepared and preincubated for 15 min at room temperature. The appropriate radiolabeled amino acid was then added to the reaction mixture and the temperature raised to 65 °C. After 1 min of incubation, the reaction was started with the addition of 5 mM ATP. Aliquots of reaction mixture were spotted on 3MM filter paper presoaked in 5

% TCA (w/v) at required time intervals, washed in 5 % TCA, dried and level of radioactivity was determined by scintillation counting. Experiments to determine the effect of EF1A on aminoacylation were performed as described above, except that *T. kodakarensis* EF1A (15 μM) was first activated by incubation at 37 °C for 30 min in a buffer containing 20 mM Tris HCl (pH 7.5), 1 mM GTP, 7 mM $MgCl_2$, 100 mM KCl, 5 mM DTT, 3 mM PEP and 30 mg/ml pyruvate kinase.

2.2.4 Polysome Preparation

T. kodakarensis cells (KW128) were grown to mid exponential phase (OD_{600} = 0.4) at 85 °C in MA-YT medium supplemented with 5g sodium pyruvate/l, and mupirocin was then added to a final concentration of 12 μg/ml and incubated at 85 °C for 5 min. Cell cultures were then chilled on ice, centrifuged at 10,000 g for 5 min at 4 °C, the resulting pellet resuspended in 0.5 ml chilled cell lysis buffer [10 mM Tris-acetate buffer (pH 7.5), 14 mM magnesium acetate, 60 mM potassium acetate and 1 mM DTT] and frozen in liquid nitrogen followed by three freeze thaw cycles. 1 μl of RNase inhibitor (Roche) was added to the lysed cells, which were then centrifuged at 16,000xg for 10 min at 4 °C and 0.25 ml of the resulting supernatant was loaded onto an 11 ml 10-40 % sucrose gradient containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NH_4Cl and 2 mM DTT. Sucrose gradients were spun in an ultracentrifuge using an SW-41 rotor at 150,000 x g for 4 h at 4°C. Gradients were fractionated using an ISCO model 183 syringe-pump at 1.48 ml/min while monitoring absorbance at 254 nm.

2.3 Results

2.3.1 *T. kodakarensis* contains an MSC that enhances tRNA aminoacylation

Previous studies identified an MSC from *M. thermautotrophicus* that contained LeuRS as a core scaffolding protein. To determine if an MSC exists in *T. kodakaraensis*, a plasmid expressing His_6-HA-LeuRS was used to produce bait for affinity co-purification experiments (Fig 2.1). Cell-free lysates of *T. kodakaraensis* / pHis_6-HA LeuRS were loaded onto a Ni^{2+} matrix, washed, and bound proteins were then eluted using an imidazole gradient. His_6-HA-LeuRS-containing fractions were identified using anti-HA antibodies, and the corresponding eluates further analyzed by mass spectrometry. A substantial number of components of the translation machinery were found associated with LeuRS including five aaRSs (TyrRS, ProRS, GlyRS, MetRS and CysRS), EF1A, IF2, IF2B, EF2 and several ribosomal proteins (Table 2.1). Consistent with previous reports describing interactions between the MSC and other pathways outside translation in archaea and eukaryotes (231, 239), proteins involved in metabolism, transcription and protein modification were also found associated with LeuRS (Table 2.2). As this analysis was performed with cells grown to mid-log phase, and given that ribosomal proteins were abundant in the interactome, some of the detected proteins may have co-purified as nascent polypeptides and may not reflect direct interactions with LeuRS.

Table 2.1. Identification of components of the protein synthesis machinery that interact with LeuRS

ORF [a]	Description [b]
TK0568	**tyrosyl-tRNA synthetase**
TK0978	**glycyl-tRNA synthetase**
TK0550	**prolyl-tRNA synthetase**
TK1461	**leucyl-tRNA synthetase**
TK0444	**cysteinyl-tRNA synthetase**
TK1049	**methionyl-tRNA synthetase**
TK0308	**elongation factor 1A**
TK0556	**translation initiation factor 2B subunit**
TK1305	**translation initiation factor 2**
TK0309	**elongation factor 2**
TK0506	**translation-associated GTPase**
TK1254	**30S ribosomal protein S3Ae**
TK1529	**30S ribosomal protein S4e**
TK1538	**30S ribosomal protein S19P**
TK1276	**30S ribosomal protein S19e**
TK1504	**30S ribosomal protein S11P**
TK1505	**30S ribosomal protein S4**
TK1496	**30S ribosomal protein S2**
TK0307	**30S ribosomal protein S10P**
TK1521	**30S ribosomal protein S5P**
TK1526	**30S ribosomal protein S8P**
TK1099	**30S ribosomal protein S27e**
TK1500	**30S ribosomal protein S9P**
TK1078	**30S ribosomal protein S12P**
TK1251	**30S ribosomal protein S15P**
TK1532	**30S ribosomal protein S17P**
TK1951	**30S ribosomal protein S6e**
TK0187	**glutamine amidotransferase, class I**
TK1239	**peptide chain release factor 1**
TK1671	**tRNA-modifying enzyme**
TK0981	**N2, N2-dimethylguanosine tRNA**
TK0970	**N(2),N(2)-dimethylguanosine tRNA**
TK0704	**SAM-dependent methyltransferase**

[a] Open reading frames (ORFs) are numbered according to the annotated genome sequence of *T. Kodakaraensis* (240). Description of the corresponding proteins are taken from the TIGR Comprehensive Microbial Resource (cmr.tigr.org/tigr-scripts/CMR/shared/AnnotationSearch.cgi).

Table 2.2. Complete list of proteins that co-purified with His_6-HA tagged LeuRS

Function	ORF	Description
Translation	TK0568	tyrosyl-tRNA synthetase
	TK0978	glycyl-tRNA synthetase
	TK0550	prolyl-tRNA synthetase
	TK1461	leucyl-tRNA synthetase
	TK0444	cysteinyl-tRNA synthetase
	TK1049	methionyl-tRNA synthetase
	TK0308	elongation factor 1-alpha
	TK0556	translation initiation factor IF-2B subunit beta
	TK1305	translation initiation factor IF-2
	TK0309	elongation factor EF-2
	TK0506	translation-associated GTPase
	TK1254	30S ribosomal protein S3Ae
	TK1529	30S ribosomal protein S4e
	TK1538	30S ribosomal protein S19P
	TK1276	30S ribosomal protein S19e
	TK1504	30S ribosomal protein S11P
	TK1505	30S ribosomal protein S4
	TK1496	30S ribosomal protein S2
	TK0307	30S ribosomal protein S10P
	TK1521	30S ribosomal protein S5P
	TK1526	30S ribosomal protein S8P
	TK1099	30S ribosomal protein S27e
	TK1500	30S ribosomal protein S9P
	TK1078	30S ribosomal protein S12P
	TK1251	30S ribosomal protein S15P
	TK1532	30S ribosomal protein S17P
	TK1951	30S ribosomal protein S6e
	TK1239	peptide chain release factor 1
	TK1671	tRNA-modifying enzyme
	TK0981	N2, N2-dimethylguanosine tRNA methyltransferase
	TK0970	N(2),N(2)-dimethylguanosine tRNA methyltransferase
	TK0704	SAM-dependent methyltransferase
	TK1114	ribonuclease Z
Proteasome	TK2252	proteasome-activating nucleotidase
	TK1637	proteasome subunit alpha

Continued

Table 2.2 Continued

Protein binding and modification	**TK2303**	**chaperonin beta subunit**
	TK1473	**amino acid kinase**
	TK1085	**protein disulfide oxidoreductase**
	TK1022	**D-aminopeptidase**
Metabolic enzymes	**TK0914**	**3-hydroxy-3-methylglutaryl-CoA reductase (mevanolate Pathway)**
	TK1470	**isopentenyl pyrophosphate isomerase (mevanolate Pathway)**
	TK0180	**acetyl-CoA acetyltransferase (mevanolate Pathway)**
	TK0828	**NAD(P)H:rubredoxin oxidoreductase (Fatty acid metabolism)**
	TK0187	**glutamine amidotransferase, class I**
	TK1404	**phosphomannomutase-related protein**
	TK0217	**pyridoxine biosynthesis protein**
	TK0890	**thiamine monophosphate kinase**
	TK1990	**cysteine desulfurase**
	TK1796	**glutamine synthetase**
	TK0193	**GMP synthase subunit B**
	TK0504	**aspartate racemase**
	TK0297	**L-aspartate oxidase**
	TK1548	**serine--glyoxylate aminotransferase, class V (transferase-transaminase)**
	TK1379	**glycine dehydrogenase subunit 2**
	TK0149	**Pyruvoyl-dependent arginine decarboxylase**
	TK2257	**deoxycytidylate deaminase**
	TK0305	**uridylate kinase**
	TK1517	**adenylate kinase**
	TK1514	**cytidylate kinase**
	TK2196	**aspartate carbamoyltransferase catalytic subunit**
	TK1895	**5'-methylthioadenosine phosphorylase II**
	TK1482	**5'-methylthioadenosine phosphorylase**
	TK1193	**CTP synthetase**
	TK2042	**ATPase**
	TK1326	**NAD(P)H-flavin oxidoreductase**
	TK1299	**NADH oxidase**
	TK1056	**rubrerythrin-related protein**
	TK0814	**Type A Flavoprotein**
	TK2290	**ribulose bisophosphate carboxylase**

Continued

Table 2.2 Continued

	TK1426	**ribose-5-phosphate isomerase A**
	TK2164	**fructose-1,6-bisphosphatase**
	TK0866	**cofactor-independent phosphoglycerate mutase**
	TK2106	**phosphopyruvate hydratase**
	TK1396	**glycerol kinase**
	TK1125	**2-oxoglutarate ferredoxin oxidoreductase subunit alpha**
	TK1123	**2-oxoglutarate ferredoxin oxidoreductase subunit gamma**
	TK1009	**putative 5-methylcytosine restriction system, GTPase subunit**
	TK1325	**Putative oxidoreductase**
	Tk1326	**ferredoxin--NADP(+) reductase subunit alpha**
	TK0793	**GTP cyclohydrolase**
	TK0893	**pyruvate formate lyase family activating protein**
	TK1984	**pyruvate ferredoxin oxidoreductase subunit beta**
	TK1205	**PBP family phospholipid-binding protein**
	TK2077	**4Fe-4S cluster-binding protein**
	TK1611	**metal-dependent hydrolase**
	TK1997	**hydrogenase maturation protein HypF**
	TK1234	**lipoate-protein ligase A, C-terminal section**
	TK0494	**bifunctional carboxypeptidase/aminoacylase**
	TK0296	**quinolinate synthetase**
	TK0868	**bis(5'-adenosyl)-triphosphatase**
	TK1549	**predicetd ATPase**
	TK1481	**NADH:polysulfide oxidoreductase**
	TK1863	**N6-adenine-specific DNA methylase**
	TK1457	**phosphate transport regulator**
	TK2016	**iron-molybdenum cofactor-binding protein**
	TK2225	**molybdenum cofactor biosynthesis protein A**
	TK0134	**acetylpolyamine aminohydrolase**
	TK1149	**methylmalonyl-CoA mutase, N-terminus of large subunit**
	TK1605	**metallo-beta-lactamase superfamily hydrolase**
	TK1716	**cytidylyltransferase**
	TK0354	**putative molybdenum cofactor biosynthesis protein C**
	TK0828	**NAD(P)H:rubredoxin oxidoreductase**
	TK1711	**sugar-phosphate nucleotydyltransferase**
Cell Division	**TK2271**	**cell division protein FtsZ**

Continued

Table 2.2 Continued

Transcription	**TK1331**	**Lrp/AsnC family transcriptional regulator**
	Tk1881	**ArsR family transcriptional regulator**
	TK1083	**DNA-directed RNA polymerase subunit beta**
	TK1503	**DNA-directed RNA polymerase subunit D**
	TK1769	**transcriptional regulator**
DNA repair	**TK2213**	**Bipolar DNA helicase**
	TK1899	**DNA repair and recombination protein RadA**
	TK0155	**RecJ-like exonuclease**
	TK1165	**AP endonuclease (base excision repair pathway)**
Ribosome Biogenesis	**TKO566**	**DEAD/DEAH box RNA helicase**
	TK0679	**serine/threonine protein kinase**
	TK1636	**putative RNA-associated protein**
Quorum sensing	**TK1605**	**metallo-beta-lactamase superfamily hydrolase**
Miscellaneous	**TK1764**	**N-acetylchitobiose deacetylase**
	TK0038	**flagellin**
	TK1037	**calcineurin superfamily metallophosphoesterase**
	TK0008	**DNA methylase**
	TK0798	**DNA topoisomerase VI subunit A**
	TK0799	**DNA topoisomerase VI subunit B**
	TK2213	**bipolar DNA helicase**
Hypothetical Proteins	**TK1492**	
	TK2200	
	TK0453	
	TK0675	
	TK1430	
	TK2148	
	TK0033	
	TK0205	
	TK1196	
	TK1424	
	TK0027	
	TK0013	
	TK1497	
	TK0175	
	TK0897	
	TK1996	
	TK0593	

Continued

Table 2.2 Continued

	TK0361	
	TK0109	
	TK0515	
	TK1626	
	TK0335	
	TK0733	
	TK0318	
	TK0206	
	TK0438	
	TK0790	
	TK2038	
	TK2294	
	TK1882	

[a] Open reading frames (ORFs) are numbered according to the annotated genome sequence of *T. Kodakaraensis* (240).

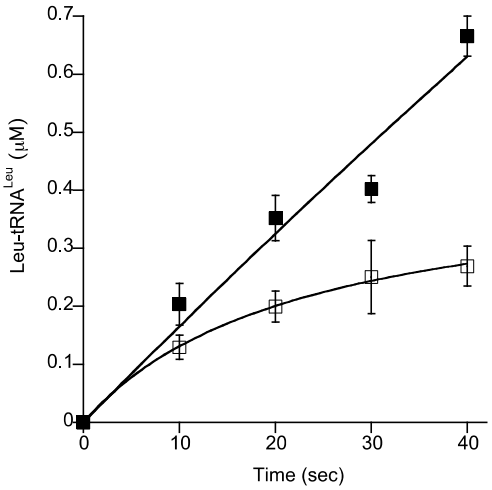
[b] Description of the corresponding proteins are taken from the TIGR Comprehensive Microbial Resource (cmr.tigr.org/tigr-scripts/CMR/shared/AnnotationSearch.cgi).

The effects of MSC formation on the aminoacylation activities of several of the corresponding aaRSs were further investigated *in vitro*. Functional effects of association between LeuRS, TyrRS, ProRS and EF1A were investigated by monitoring the aminoacylation activities of the aaRSs in the presence of other enzymes. An excess of each partner protein was used in order to minimize the free fraction of the monitored enzyme. Addition of a 10-fold excess of ProRS increased the aminoacylation activity of LeuRS approximately 2-fold; a 40-fold excess was necessary to produce a comparable change in the presence of TyrRS, suggesting this aaRS forms a weaker interaction with LeuRS than ProRS (Fig. 2.3). The possible enhancement of aminoacylation activities of ProRS and TyrRS in the presence of excess partner aaRSs was also investigated, but no

significant differences were observed compared with the activities of the respective enzymes alone. Previously, it has been shown in archaea and eukaryotes that addition of EF1A increases the activity of LeuRS and ValRS, respectively (79, 224), promoting us to investigate if this was also the case for *T. kodakarensis* LeuRS. The GDP-bound form, as well as the GTP activated state were tested for their possible effects on the aminoacylation activity of LeuRS. The largest increase in the rate of Leu-tRNA^{Leu} formation was observed in the presence of EF1A·GTP, indicating that enhancement of LeuRS activity depends on the presence of activated elongation factor (Fig. 2.4). The inclusion of more than two partners, for example the co-incubation of LeuRS, ProRS and EF1A together, did not further enhance aminoacylation rates above those observed when only two partners are present.

MetRS and CysRS were not included in these experiments as they did not co-associate with other putative MSC components during polysome analyses (see *3.2* below), and GlyRS was also excluded as the recombinant protein was not active when tested for *in vitro* aminoacylation activity (data not shown).

A



B

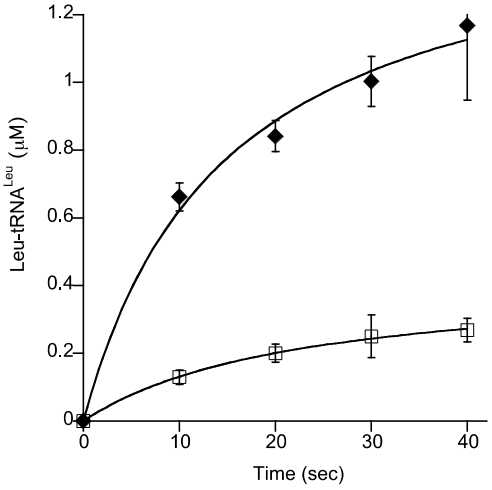


Figure 2.3. Effects of ProRS and TyrRS on aminoacylation by LeuRS. *T. kodakarensis* total tRNA (2 mg/ml) was aminoacylated under standard conditions (7 μl samples) in the presence of LeuRS alone (80 nM) (□) or with addition of **(A)** ProRS (800 nM) (■) or **(B)** TyrRS (3.2 μM) (◆). Non-specific increase in the activity of aaRSs were excluded by adding BSA in the reaction mixtures. Further addition of BSA did not change the activities of the aaRSs being monitored (data not shown).

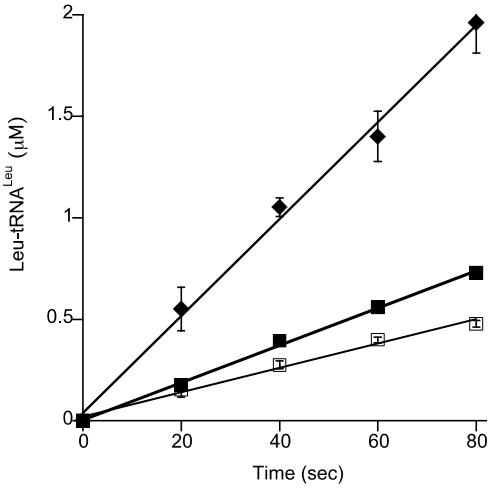


Figure 2.4. Effect of EF1A on aminoacylation by LeuRS. *T. kodakarensis* total tRNA (2 mg/ml) was aminoacylated under standard conditions (7 μl samples) in the presence of LeuRS alone (15 nM) (□), or with addition of EF1A-GTP (3.5 μM) (◆) or EF1A-GDP (3.5 μM) (■).

2.3.2 Association of the MSC with the protein synthesis machinery

Affinity co-purification of proteins interacting with LeuRS identified several ribosomal proteins, suggesting that the archaeal MSC might interact with actively translating ribosomes. To investigate whether aaRSs are associated with actively translating ribosomes in *T. kodakarensis*, we attempted to isolate polysomes from exponentially growing cells and subsequently separate them from other ribosomal fractions by sucrose gradient sedimentation. Several antibiotics were tested for their ability to stall translation in *T. kodakarensis* cells, a requirement for stable polysome isolation, and of these mupirocin was found to be the most effective (Fig. 2.5.A). Mupirocin is a polyketide antibiotic produced by *Pseudomonas fluorescens* that inhibits archaeal isoleucyl-tRNA

synthetase *in vivo*, and it was used here to facilitate polysome isolation from *T. kodakarensis* (241, 242). Polysomes, monosomes and individual small and large ribosomal subunits were fractionated over sucrose density gradients, and the distribution of tRNA aminoacylation activities associated with each fraction then investigated. The distribution of LeuRS was examined in detail, and was found to be enriched in polysomes and to a lesser extent other ribosomal fractions as compared to non-ribosomal fractions (Fig. 2.5.B). The presence of other aaRSs in ribosomal fractions was investigated by monitoring activities associated with the ribosomal 50S ribosomal fraction, which was chosen due to its relatively high abundance, middle position among the ribosomal fractions, and good separation from non-ribosomal fractions. Seven aaRSs out of the ten tested were found enriched in the ribosomal fraction, as compared to the non-ribosomal fraction S1 (Fig. 2.5C). Among the 7 aaRS activities identified were ProRS, LeuRS and TyrRS, which were also shown to associate in *T. kodakarensis* by affinity co-purification (see above), providing further support for the direct association of the MSC with actively translating ribosomes. The other aaRSs found to be associated with ribosomes might also be present in the MSC, but may have escaped detection by mass spectrometry due to the inherent limitations of pulling down large complexes using an epitope-tagged bait protein.

A

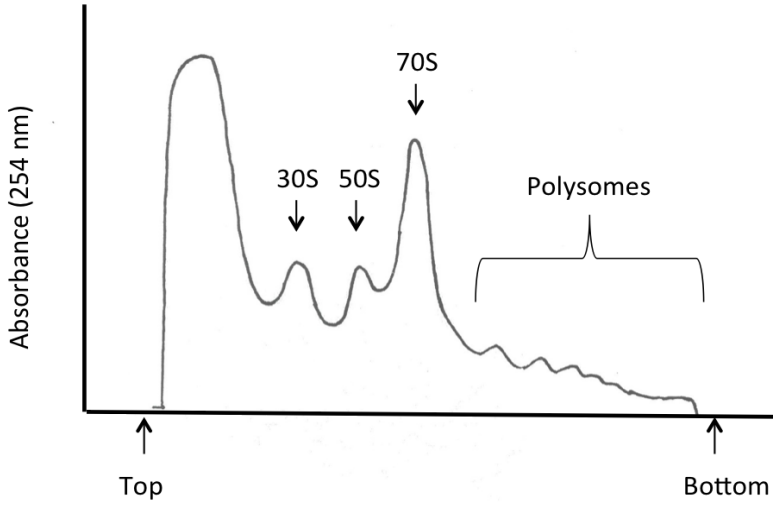
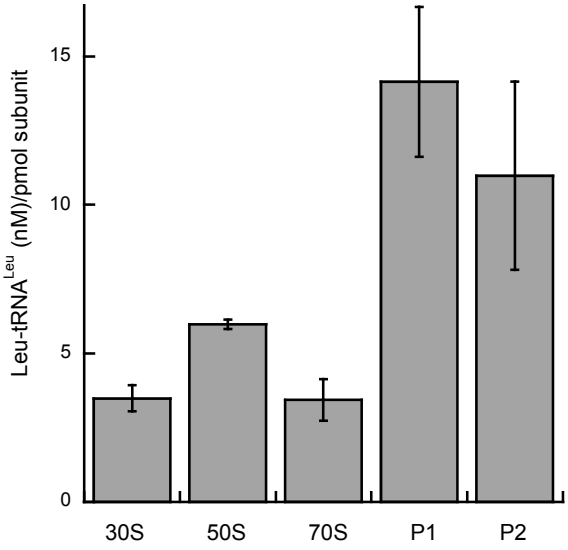


Figure 2.5. Association of aminoacylation activities with the protein synthesis machinery. *T. kodakarensis* cells were grown under anaerobic conditions until mid log phase and then subjected to polysome analysis. Polysome lysates were fractionated by sucrose gradient centrifugation and the ribosomal subunits, monosomes and polysome fractions collected. **(A)** absorbance profile (A_{254}) of fractions eluted from the gradient. The top of the gradient is on the left and peaks representing the 30S and 50S ribosomal subunits, monosomes and polysomes are denoted. **(B)** Leu-tRNA aminoacylation activity in ribosomal subunits, monosomes and polysomes. **(C)** Aminoacylation activities associated with 50S ribosomal subunits.

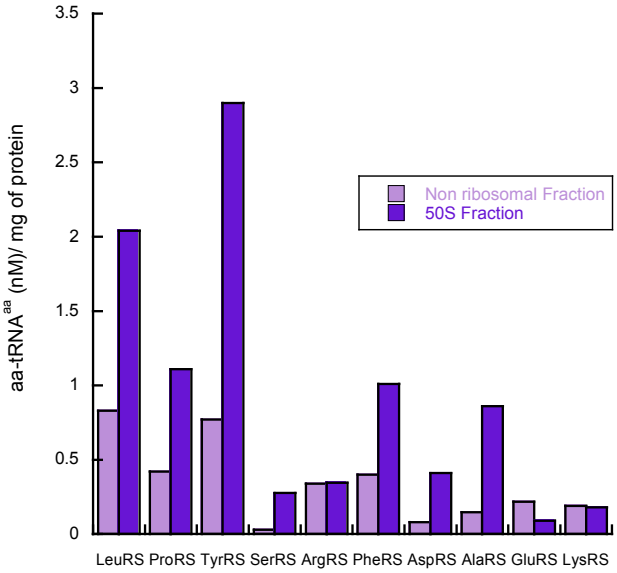
Continued

Figure 2.5 Continued

B



C



2.4 Discussion

In the present study the LeuRS interactome of *T. kodakarensis* was determined using a combination of co-affinity purification and proteomics. The interactome contained, among other proteins, 5 aaRSs (ProRS, TyrRS, GlyRS, MetRS and CysRS), EF1A, EF2, IF2B and IF2, and a number of ribosomal proteins. LeuRS, ProRS and EF1A have previously been shown to be part of a complex in *M. thermautrophicus*, while ProRS, MetRS and LeuRS are components of the mammalian MSC (223). Interactions between *T. kodakarensis* LeuRS with TyrRS or ProRS enhanced $tRNA^{Leu}$ aminoacylation, similar to previous reports of improved aminoacylation upon aaRS complex formation in archaea and yeast (80, 83, 227). The catalytic enhancement observed here was less extensive than previously described in *M. thermautrophicus*, being limited to LeuRS with no comparable improvements observed in the aminoacylation activities of TyrRS or ProRS. LeuRS activity was also enhanced by the GTP-activated form of archaeal EF1A but not by GDP-bound EF1A. EF1A-GTP preferentially binds aa-tRNA (243), and the observed enhancement is consistent with previous reports of eukaryotic aaRSs forming specific interactions with elongation factors (224, 244). The ability of activated elongation factors to increase aaRS product release rates was previously proposed to accelerate aa-tRNA synthesis (245), and our findings now suggest this to be the case for Leu-$tRNA^{Leu}$ in the *T. kodakarensis* MSC.

Enhanced aa-tRNA synthesis within MSCs provides a possible mechanism to increase protein synthesis rates, as does the association of such complexes directly with ribosomes (246). The presence of 16 30S ribosomal proteins, IF2, IF2B and EF2, in the

LeuRS interactome, and the detection of several aaRS activities in polysomes, both support the association of the identified MSC with the translation machinery. The detection of IF2, IF2β, MetRS and EF2 in the interactome along with 30S, but not 50S, ribosomal proteins suggests the MSC might first interact with the 30S preinitiation complex or that protein-protein interactions are not sufficiently persistent to survive our purification procedures. Kang *et al.* recently showed that AIMP3, the nonenzymatic scaffolding protein of the mammalian MSC interacts with Met-$tRNA_i^{Met}$ and the eIF2 complex and plays an important role in translational initiation by mediating the delivery of charged initiator tRNA to initiation complex thereby supporting the idea of possible interaction of the MSC with the translation initiation complex (247). Conversely, a MSC containing a methanogenic-type SerRS and ArgRS was shown to interact with the 50S L7/L12 stalk in *M. thermautrophicus* suggesting a mechanism of tRNA recycling in which aaRSs associate with the L7/L12 stalk region to recapture the tRNAs released from the preceding ribosome in polysomes (248). The observation of interaction of MSC with different ribosomal subunits raises the question of whether the synthetases that are part of the MSC stably interact with the ribosoma subunits, or instead form more transient interactions with them. A more detailed structural analysis of the protein–protein interactions between tRNA synthetases and the ribosome may yield further insights into the mechanistic features of these interactions.

While the precise details of some of the interactions remain to be resolved, as does the possible structural role of tRNA (83), our data indicate the presence in *T. kodakarensis* of a larger macromolecular assembly including the MSC, the translational

machinery, and perhaps other major complexes such as the proteasome, as also recently observed in *Schizosaccharomyces pombe* (239). The channeling of aa-tRNAs directly to ribosomes without diffusion into the cytoplasm has been proposed to improve translation efficiency, and the observation in *T. kodakarensis* of MSCs associated with active ribosomes is consistent with this model. Direct investigation of the importance of MSCs *in vivo* may now be possible in *T. kodakarensis* by targeted disruption of protein-protein interactions between specific aaRSs, which would also help to address long-standing questions as to the impact on the cell of tRNA channeling during translation.

Chapter 3:

Oxidation of cellular amino acid pools leads to cytotoxic mistranslation of the genetic code

3.1 Introduction

The faithful translation of mRNA into the corresponding protein sequence is an essential step in gene expression. The accuracy of translation depends on the precise pairing of mRNA codons with their cognate aminoacyl-tRNAs, containing the corresponding anticodons, during ribosomal protein synthesis (249, 250). Cognate amino acids are attached to their respective tRNAs by aminoacyl-tRNA synthetases (aaRSs), and the ability of these enzymes to distinguish between cognate and non-cognate substrates is a major determinant of the fidelity of the genetic code. AaRSs discriminate against near- and non-cognate tRNAs at levels compatible with typical translation error rates ($\sim 10^{-4}$) due to the structural complexity and diversity observed between tRNA isoacceptors. AaRSs can less successfully discriminate against near-cognate amino acids, which may differ from the cognate substrate by as little as a single methyl or hydroxyl group. Errors

during amino acid recognition do not usually compromise the accuracy of translation due to highly specific aaRS enzymes, and the widespread existence of editing mechanisms that proofread non-cognate amino acids. For example, phenylalanine tRNA synthetase (PheRS) edits mischarged Tyr-tRNA^{Phe} at a hydrolytic editing site ~30 Å from the synthetic active site (251, 252). PheRS editing provides a key checkpoint in quality control, as mischarged Tyr-tRNA^{Phe} is readily delivered to the ribosome by EF-Tu where it can efficiently decode Phe codons as Tyr in the growing polypeptide chain, resulting in mistranslation (40, 41).

Despite their role in accurately translating the genetic code, aaRS editing pathways are not conserved, and their activities have varying effects on cell viability (54, 253-255). *Mycoplasma mobile*, for example, tolerates relatively high error rates during translation and lacks PheRS editing function, as do other aaRSs in this organism (59, 60). *Saccharomyces cerevisiae* cytoplasmic PheRS (*Sc*ctPheRS) has a low Phe/Tyr specificity and is capable of editing, whereas the yeast mitochondrial enzyme (*Sc*mtPheRS) completely lacks an editing domain, and instead relies on high Phe/Tyr specificity. *Escherichia coli,* in contrast, has retained both features and displays a high degree of Phe/Tyr specificity and robust editing activity (256). The range of divergent mechanisms used by different PheRSs to discriminate against non-cognate amino acids illustrates how the requirements for translation quality control vary with cellular physiology (59). Furthermore, given that editing by PheRS and other aaRSs is not essential for viability in yeast or *E. coli*, it is clear that the true roles of these quality control pathways remain to be fully elucidated (256, 257).

In addition to the well-documented ability of aaRSs to edit tRNAs charged with genetically encoded near cognate amino acids, these same proofreading activities have been demonstrated to act on other non-canonical substrates. AaRSs are able to edit tRNAs misacylated with a range of amino acids not found in the genetic code such as homocysteine, norleucine, α-aminobutyrate and *meta*-tyrosine (*m*-Tyr), although the physiological relevance of these activities is unknown [reviewed in (20)]. Both *E. coli* and *Thermus thermophilus* PheRS have been shown to edit *m*-Tyr, a metabolic byproduct formed by oxidation of phenylalanine following metal-catalyzed formation of hydroxyl radical species (258-260). Certain species of fescue grasses (*Festuca* spp.) produce *m*-Tyr as a natural defense agent that appears in the proteomes of neighboring plants, and *m*-Tyr accumulation in the proteome of Chinese hamster ovary (CHO) cells has been proposed to have a cytotoxic effect on translation (261, 262). Taken together, these findings suggest that oxidative stress could potentially result in *m*-Tyr accumulation with the accompanying threat of cytotoxic mistranslation. Under such growth conditions, the ability of the cell to edit *m*-Tyr-tRNA^{Phe} would be essential to maintain cellular viability. Here we investigate the requirements for bacterial and yeast PheRS editing using editing deficient mutants under normal and oxidative stress conditions.

3.2 Materials and methods

3.2.1 Strains, plasmids, and general methods

Proteins and tRNAs were prepared essentially as described previously (263). Mutation of the *E. coli* PheRS gene in the pQE31-EcFRS expression plasmid was completed using standard polymerase chain reaction (PCR)-based site-directed mutagenesis as previously described (40). Purification of His-tagged PheRS variants included dialysis against two changes of 25 mM Tris-HCl pH 7.5, 100 mM KCl, 0.1 mM sodium pyrophosphate, 3 mM 2-mercaptoethanol, and 10% glycerol, in order to release any enzyme-bound adenylate. Dialysis against 25 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 10% glycerol was then performed followed by dialysis against this same buffer with 50% glycerol, flash frozen, and stored at -80^{o}C. Active enzyme concentration was determined by active site titration as previously described (264). Phenylalanine, L-*p*-tyrosine and D,L-*m*-tyrosine were purchased from Sigma Aldrich.

3.2.2 Construction of editing defective *E. coli* and yeast mutant strains

The editing deficient strain of *E. coli*, *pheT(G318W)*, was constructed using established recombineering methods involving the lambda red/gam pKD46 plasmid (265). The *pheS*^{ts} *E. coli* strain NP37, which contains a G98D mutation (266) was used as the parental strain to allowed for selection of recombination events within the region of the *pheS* and *pheT* genes. Site directed mutagenesis of the pQE31-EcFRS wt plasmid (40) was used to construct pQE31-EcFRSG318W/V364V. Linear PCR products were

amplified from this plasmid and introduced to the pKD46 containing NP37 parent strain via electroporation. Primers for PCR were as follows: p14 EcFRS: 5'-AACCATGTCACATCTCGC and P16AS EcFRS: 5'-CGTTGGTGATATCAATTA CCGG. This linear DNA contains the wild-type *pheS* gene to allow for colony selection at 42^{o}C, the *pheT* gene containing a G318W mutation, and a silent V364V mutation that introduces a BamHI site for screening of colonies. Recombinant strains were confirmed with sequencing. A wild-type *pheS/pheT* strain was also constructed in the same manner, but without changing the Gly residue at 318. The λ-red recombineering system was used to introduce the *pheT(G318W)* mutation into the *E. coli* MG1655 background. Competent cells were prepared as previously described (267) of an MG1655 derivative containing pSIM6, a plasmid that carries the λ-red system (268). These cells were transformed with a 70-mer oligonucleotide (5'- CACAACAAGGCGCTGGCGATGGG AGGAATATT TTGGGGAGAGCATTCAGGCGTGAAT GACGAAACACAAA) that has several wobble mutations (underlined) on either side of the *pheT(G318W)* mutation (bolded). The wobble mutations serve to overwhelm the mismatch repair system (269). Positive clones were identified by colony PCR, with a primer that recognized the mutated sequence (5'-AGGAATAT TTTGGGGAGAGCATTCA) and a reverse primer 500-bp distant (5'-CCGATCAGGCGATCC AGTTTG), and subsequent DNA sequencing. One clone was chosen to serve as the intermediate strain and was subjected to a second round of recombineering, as indicated above, with an oligo (5'CACAACAAGGCGCTGGCGA TGGGCGGCATCTTCTGGGGCG AACACTC TGGCGTGAATGACGAAACACAAA) to remove the wobble mutations and leave solely the *pheT(G318W)* mutation. The

intermediate strain was also transformed with an oligo (5'-CACAACAAGGCGCTGGCGATGGGCGGCATCTTCGGTGGCGAACACTCTGGCG TGAATGACGAAACACAAA) that would revert the strain back to the wild-type *pheT* sequence. This strain served as the wild-type control strain in studies with the *pheT(G318W)* derivative of *E. coli* MG1655. Again, positive clones were screened by colony PCR (primer 5'-CGGCATCTTCTGG GGCGAACACTCT for *pheT(G318W)* and primer 5'-CGGCATCTTCGGTGGCGAACACTCT for wild-type, both with the reverse primer indicated above) and DNA sequencing. Strains derived from *S. cerevisiae* W303 (*MATa/MATα*, *ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*) were used to construct chromosomal mutants of *FRS1*. A 2084 bp fragment of *frs1-1*, obtained through PCR of the plasmid pFL36-*frs1-1* (257), was inserted into the integrative plasmid YIP5 (270) at the EcoRI and NruI restriction sites by In-Fusion cloning (Clontech), resulting in the plasmid YIP5-*frs1-1*. W303 (*MATa/MATα*, *ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*) was transformed with YIP5-*frs1-1* and insertion of the plasmid was selected for by growth on complete supplement media minus uracil (CSM -Ura; Sunrise Science Products). Recombinant strains were grown in YPDA at 30 °C, shaking at 300 rpm, for 24 h, and plated on YPDA. Crossovers were selected for by replica plating onto media containing 5-flouroorotic acid (5-FOA). *TRP1* prototroph strains were created through the PCR amplification of the *TRP1* locus from *S. cerevisiae* strain BY4743 (*MATa/MATα, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0*) and the linear product used to transform the W303 (*MATa/MATα, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, FRS1/frs1-1,*) strain. *TRP1*

recombinants were selected on synthetic complete minus tryptophan media. Haploids were obtained by sporulation, dissection onto YPDA, replica plated onto complete supplement media minus tryptophan, and tryptophan prototroph colonies selected. Haploids were screened for the presence of the *frs1-1* mutation, resulting in the strains NR1 (*MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, can1-100*) and NR2 (*MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, can1-100, frs1-1*).

3.2.3 Growth assays

Single colonies of *E. coli*, wild type *pheT* or *pheT(G318W)*, were picked from LB plates, resuspended in sterile water and used to inoculate liquid culture at an initial OD_{600} of 0.04. Cultures were grown in M9 media supplemented with glucose (2 g/l), thiamine (1 mg/l), $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM), and varying amounts of amino acids. Cultures were grown at 37^{o}C in 250 µl volumes using 96-well plates for ease of titrating several amino acid concentrations. Phe was kept constant at 0.003 mM and L-Tyr or D,L-*m*-Tyr was varied from 0.003 mM to 3 mM. Optical densities at 600 nm (OD_{600}) were read using a xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories) after 12-18 hours of growth. Growth curves were performed in supplemented M9 media containing none or 0.5 mM *D,L-m*-Tyr, and 100 ml cultures were grown at shaking at 37^{o}C. Growth experiments in the presence of oxidative stress agents were also set up in 96-well plates in M9 minimal media containing 0.5 mM Phe, 0.1 mM $FeSO_4$, and 2-4 mM H_2O_2. For all growth assays of the *S. cerevisiae* strains NR1 and NR2, cells were streaked on YPDA

and incubated at 30 °C. After approximately 72 h single colonies were picked, resuspended in sterile water and used to inoculate liquid cultures to an initial OD600 of 0.01. Microtitre growth assays were completed by inoculating 150 μL of MM (Difco™ yeast nitrogen base without amino acids, 0.002% adenine, 0.002% uracil, 0.002% L-histidine, 0.01% L-leucine, and 2% glucose) + Phe:Tyr (where Phe was kept constant at 0.003 mM and Try was varied from 0.003 mM to 1.2 mM) in a 96 well microtitre plate. Plates were incubated at 30 °C and growth was measured after 16 h by OD600.

3.2.4 *S. cerevisiae* aging assays

S. cerevisiae aging assays were completed using the methods of Fabrizio and Longo (271). The *S. cerevisiae* strains NR1 and NR2 were streaked on YPDA and incubated at 30 °C. After approximately 72 h several colonies were picked and resuspended in sterile water and used to inoculate 50 mL of synthetic complete medium (SDC) with variable Phe:Tyr ratios in a 250 mL flask to an initial OD600 of 0.1. Cultures were grown in SDC + Phe:Tyr 1:1 where Phe was at a concentration of 0.003 mM. SDC media consisted of 0.18 % yeast nitrogen base without amino acids and ammonium sulfate, 0.5 % ammonium sulfate, 0.14 % NaH2PO4, 80 mg/L adenine, 80 mg/L uracil, 80 mg/L tryptophan, 80 mg/mL histidine-HCl, 40 mg/L arginine-HCl, 80 mg/L methionine, 1200 mg/L leucine, 60 mg/L isoleucine, 60 mg/L lysine-HCl, 100 mg/L glutamic acid, 100 mg/L aspartic acid, 150 mg/L valine, 200 mg/L threonine, and 400 mg/L serine. Cultures were shaken at 220 rpm at 30 °C for 72 h, after which samples of 1 mL were taken every

48 h, serial dilution performed in sterile water, and plated on YPDA. Plates were allowed to grow for 2-3 d at 30 °C, colonies counted, and CFU/mL calculated for each culture. Assays were performed in triplicate.

3.2.5 Northern blot analysis

Strains NR1 and NR2 were inoculated into 50 mL liquid YPDA, minimal media (MM) + Phe:Tyr 1:1 (0.003 mM Phe, 0.003 mM Tyr), or minimal media (MM) + Phe:Tyr 1:50 (0.003 mM Phe, 0.15 mM Tyr) and grown to an OD_{600} of 0.8. Cells were harvested by centrifugation, washed with 50 mL dH_2O, frozen, and stored at -80°C overnight for RNA extraction. The unfolded protein response was induced by treating cultures at an OD_{600} of 0.4 with Tunicamycin (10 μg/mL), grown to OD_{600} of 0.8 and processed as above. Total cellular RNA was extracted using hot phenol. Northern blot analysis was carried out using the NorthernMax –Gly Kit (Ambion) according the manufacturer's instructions. Agarose gels were loaded with 5 μg of total RNA for each sample. A single stranded DNA probe (5'-CAAACAAATTGTTGTTGT CTACGGCAGGTAG-3') which binds to base pairs 529-559 of the *HAC1* transcript was radiolabeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase and utilized in hybridization.

3.2.6 tRNA preparation and ^{32}P labeling

Purified native *E. coli* $tRNA^{Phe}$ was purchased from Chemical Block, Moscow.

S. cerevisiae cytoplasmic and mitochondrial $tRNA^{Phe}$ were made from T7 runoff transcription as previously described (23, 263). DNA template for tRNA transcription was generated from plasmids carrying tRNA genes (272) by PCR amplification and extended only to C75 to allow for ^{32}P labeling of A76. After ethanol precipitation, tRNA transcripts were purified on denaturing 12% polyacrylamide gel and extracted by electrodialysis in 90 mM Tris-borate/2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The tRNA was phenol and chloroform extracted, ethanol precipitated, dried and resuspended in diethylpyrocarbonate (DEPC)-treated ddH_2O. Refolding was carried out by heating the tRNA at 70°C for 2 min, followed by the addition of 2mM $MgCl_2$ and slow cooling to room temperature. tRNAs were ^{32}P-labeled at A76 essentially as described previously (263). For *E. coli* $tRNA^{Phe}$ the CCA-3'-end was removed prior to labeling by treatment of 20 μM tRNA transcript with 100 μg/ml *Crotalus atrox* venom (Sigma) in a buffer containing 50 mM Na-Gly (pH 9.0) and 10 mM magnesium acetate. The reaction was incubated for 40 min at 21°C and phenol/chloroform extracted, ethanol precipitated, and desalted by gel filtration through a Sephadex G25 column (Amersham Biosciences). The CCA-3'-end of the tRNA was reconstituted and radiolabeled using *E. coli* tRNA terminal nucleotidyltransferase and [α-^{32}P] ATP as described (263). Yeast cytoplasmic and mitochondrial $tRNA^{Phe}$ C75 transcripts were labeled the same way, however CTP was excluded from the reaction mix. Samples were treated with one volume of phenol, and the tRNA was phenol/chloroform extracted and gel filtered twice through a G25 column.

3.2.7 Aminoacylation and editing Assays

Aminoacylation reactions were performed at 37^{o}C in aminoacylation buffer (100 mM Na-Hepes pH 7.2, 30 mM KCl, 10 mM $MgCl_2$, 10 mM DTT) with 8 mM ATP, 60 (*E. coli*) or 100 μM (*S. cerevisiae*) cold amino acid, 0.5 μM ^{32}P-tRNA. PheRS (100 nM) was added to initiate the reactions. Aliquots were removed at designated time points, treated with an equal volume of 0.5 M sodium acetate pH 4.2 and incubated for 30 min at room temperature with S1 RNase (Promega). The free [α-^{32}P]AMP and aminoacyl-[α-^{32}P]AMP were separated by thin layer chromatography on polyethyleneimine cellulose (Sigma Aldrich) in 100 mM ammonium acetate, 5% acetic acid and visualized as described previously (273). Mischarging of *E. coli* tRNA^{Phe} was performed at 37^{o}C for 20 min in aminoacylation buffer with 8mM ATP, 100 μM cold with L-*p*-Tyr or D,L-*m*-Tyr, 4 μM ^{32}P-tRNA and 1 μM αA294G/βG318W PheRS (251). Reactions were stopped by the addition of 1 volume of phenol pH 4.5, and the aminoacylated tRNA was phenol/chloroform extracted and gel filtered twice through a G25 column pre-equilibrated with 5 mM sodium acetate pH 4.2. Editing assays were performed in aminoacylation buffer and contained 0.1 μM Tyr-[^{32}P] tRNA^{Phe}, and 10nM G318W PheRS. Reactions were arrested at various time points and analyzed by TLC as described for the aminoacylation reactions (see above). Editing assays of the cell-free extracts were performed similarly, however mischarged [^{14}C]Tyr-tRNA^{Phe} was formed (263), and 1 μM was used in reactions containing aminoacylation buffer, 2 mM ATP, and cell free extract that was normalized for aminoacylation activity.

3.2.8 ATP/PP_i exchange

ATP/PP_i exchange assays were performed according to standard methods as previously described (251, 263). Reactions were carried out at 37°C in a medium containing 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 2 mM [^{32}P]PP_i (2 cpm/pmol), varying amounts of Phe (1-200 µM) and D,L-*m*-Tyr (20 to 2000 µM), and 40 nM *E. coli* PheRS, 100-150 nM yeast cytosolic enzyme. After 1–1.3 min, 25 µl of the reaction were removed and added to a solution containing 1% charcoal, 5.6% $HClO_4$, and 75 mM PP_i. The charcoal-bound ATP was filtered through a 3MM Whatman filter discs under vacuum and washed three times with 5ml of water and once with 5ml of ethanol. The filters were dried, and the radioactivity content was determined by liquid scintillation counting. We previously reported the activation specificity of Phe versus *p*-Tyr to be 7800 (257), however this discrepancy appears to be due to differences in enzyme-bound aminoacyl adenylate during protein purification affecting the measured active enzyme concentration. This problem was resolved here through extensive dialysis against PP_i.

3.2.9 Dipeptide synthesis

Initiation complexes (70S IC) were formed using tight coupled 70S ribosomes, [^{35}S]fMet-$tRNA^{fMet}$, Met-Phe coding mRNA, and initiation factors essentially as described (274). Ternary complexes were formed using aminoacylated $tRNA^{Phe}$ and activated EF-Tu (274). Reactions were initiated by mixing 1 µM ternary complex with 0.1 µM 70S IC

and incubated for 1 min at 21^{o}C before quenching with 1/5th volume of 2 M KOH and 1M H_2O_2. Quenched reactions were then incubated at 37^{o}C for 20 minutes to deacylate $tRNA^{Phe}$, and [^{35}S]fMet-Phe dipeptides were separated from [^{35}S]fMet by TLC on silica plates in buffer containing 1-butanol:acetic acid:H_2O (4:1:1). TLC plates were then exposed and quantified by phosphor imaging.

3.2.10 Quantification of amino acid pools

Cultures were grown to late log phase in supplemented M9 media with or without 0.5 mM tyrosine in 5 ml volumes and harvested by vacuum filtration over a nylon filter followed by washing cells three times with 1 ml H_2O. Cells and filters were then placed upside down in 0.5 ml extraction buffer (40% acetonitrile, 40% methanol) containing internal standards (100 pmol [U^{13}C]Phe and 100 pmol [U^{13}C]Tyr) at -20^{o}C for 15 minutes. Metabolites were extracted as described (275) and vacuumed dried. Samples were re-dissolved in water (50 ul), centrifuged (16,000 x g, 5 min) and the supernatant transferred to LC injector vials. Aliquots of the supernatant (typically 5 µl) were injected onto a reverse phase HPLC column (Phenomenex Kinetex XB-C18, 2.1 x 100 mm, 1.7 µm particle size, 100 Å pore size) equilibrated in solvent A (water/formic acid, 100/0.1, v/v) and eluted (100 µl/min) with an increasing concentration of solvent B (acetonitrile/formic acid, 100/0.1, v/v; min/%B, 0/1, 5/1, 26/70, 27/1, and 35/1). The effluent from the column was directly connected to an electrospray ion source (Agilent Jet Stream) attached to a triple quadrupole mass spectrometer (Agilent 6460) scanning in

the multiple reaction monitoring mode with standard resolution settings (FWHM 0.7) using previously optimized conditions for the following transitions: Tyr, 182→136; $U^{13}C$-Tyr, 191→144; Phe, 166→120; U ^{13}C-Phe, 175→128. With each batch of samples a series of standards was prepared with the same amount of internal standards and increasing amounts of Tyr and Phe (0, 0.1, 1, 10 and 100 pmol in 50 µl of water, in duplicate). Typical retention times for *p*-Tyr, *m*-Tyr, *o*-Tyr and Phe were 4.8, 6.6, 8.6 and 9.7 min, respectively. Peak areas were measured using instrument manufacturer supplied software (Agilent MassHunter). The amount of each analyte in each sample was determined by interpolation from the curves constructed from the standard samples (peak area Tyr or Phe/peak area $U^{13}C$-Tyr or –Phe against amount of Tyr or Phe).

3.2. 11 Purification and LC-MS/MS-MRM of total protein hydrolysate

E. coli cultures (100 ml), prepared in duplicate, were grown in M9 minimal media with or without 0.5 mM *m*-Tyr to exponential phase and harvested by centrifugation (6000 g, 10 minutes). Cell pellets were washed twice, resuspended in water, and lysed by sonication. To precipitate ribosomes and nucleic acids, streptomycin sulfate was added to a final concentration of 8 mg/mL (276). Samples were incubated at 4°C for one hour, then centrifuged at 11,000 g for 5 minutes. Supernatants were collected and brought to 55% acetone by volume at 4^{o}C for one hour. Precipitated material was pelleted at 11,000 g for 5 minutes. Supernatants were discarded and the pellets were washed twice with 60% acetone (ice cold). The pellets were then subjected to two methanol/chloroform

extractions, vacuumed dried, and weighed. One set of samples was used for measurement of protein content (bicinchoninic assay, Thermo Scientific). After resuspending in water and addition of internal standards ($U^{13}C$-Tyr and $U^{13}C$-Phe, 100 pmol each), the other set of samples was subjected to acid hydrolysis (6 M HCl for 24 hrs at 110^{o}C). LC-MS/MS-MRM was performed on the hydrolysate as described above.

3.3 Results

3.3.1 PheRS editing is dispensable for viability in *E. coli* and *S. cerevisiae*

To investigate the role of *E. coli* PheRS (*Ec*PheRS) editing *in vivo*, a strain was constructed containing a point mutation (G318W) within *pheT*, which encodes the β subunit of PheRS. Changes to residue βG318 hinder access to the editing site and thereby reduce *Ec*PheRS post-transfer editing activity by more than 70-fold *in vitro* (251, 277). *E. coli* strain NP37, which encodes a temperature sensitive *pheS* allele, was used as the background strain in order to facilitate selection of recombinant strains (278). Cell-free extracts from non-temperature-sensitive NP37-derived strains with wild type *pheT* and *pheT(G318W)* alleles were prepared and their PheRS activities tested. Only the strain encoding wild type PheRS retained post-transfer editing activity against *p*-Tyr-tRNA^{Phe} (Fig. 3.1A). Both strains showed identical levels of aminoacylation activity and growth at 37 °C, indicating that the proofreading pathway is not required for viability. The role of PheRS editing in maintaining cell viability was also investigated in *S. cerevisiae* by mutation of the chromosomal *FRS1* gene, which encodes the β-subunit of cytoplasmic

PheRS (*Sc*ctPheRS). Introduction in *FRS1* of a mutation encoding the amino acid replacement D243A eliminated *p*-Tyr-tRNA^{Phe} editing *in vivo* [Fig. 3.1B; (257)] and had no effect on growth and viability compared to wild type under standard conditions.

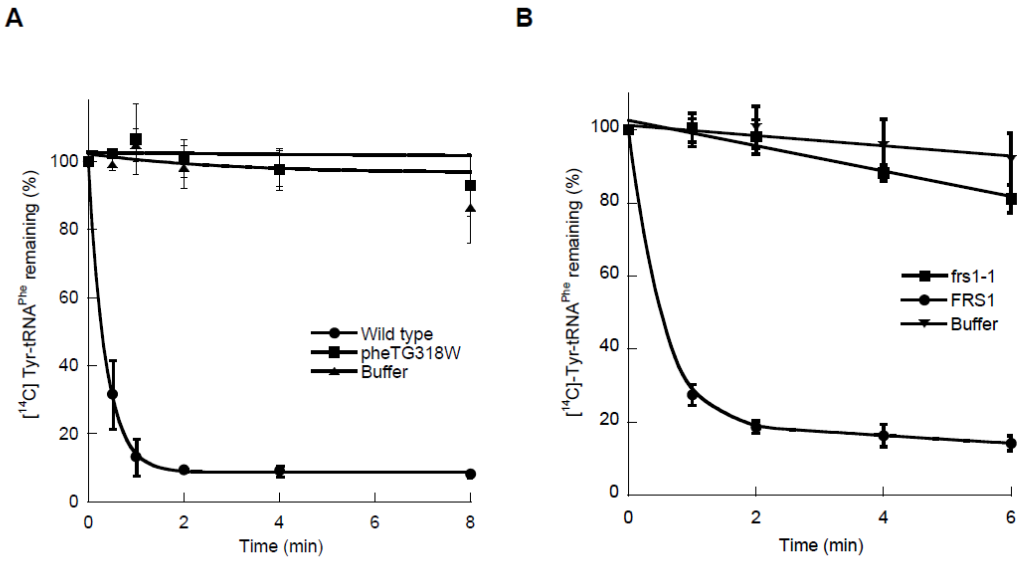


Figure 3.1. Chromosomal editing mutants of *E. coli* and *S. cerevisiae.* (A) Post-transfer hydrolysis of [^{14}C]- Tyr-tRNA^{Phe} (1μM) by cell-free extracts isolated from wild type (●) and *pheT(G318W)* (■) *E. coli* strains (140mg/ml total protein concentration) or buffer (▼) at 37^{o}C. **(B)** Post-transfer editing activity of βD243A ctPheRS in *S. cerevisiae*. Reactions were performed at 37 °C with 2 μM Tyr-tRNA^{Phe} and 0.006 U of *S. cerevisiae wild type* FRS1 or *frs1-1* (D243A) cell-free extracts extract (257). Data points are the mean of at least three independent experiments, with errors bars representing ±1 SD. Experiment performed by Dr. Tammy Bullwinkle.

3.3.2 PheRS editing specifies *m*-Tyr resistance in *E. coli*

Phenotypic microarrays (Biolog) were used to compare the growth of *E. coli pheT(G318W)* to wild type under 1920 growth conditions, and no significant changes were observed in the absence of PheRS editing. Additional experiments to investigate possible roles for editing under a range of other conditions, including heat shock, cold shock, pH stress and aging, failed to reveal differences compared to wild type. Growth of these strains was also compared in media containing varying concentrations of near-cognate *p*-Tyr in order to test the limits of *Ec*PheRS specificity in the absence of post-transfer editing activity. Elevated concentrations of *p*-Tyr (>3mM) did not affect the growth of *E. coli pheT(G318W)* compared to wild type (Fig. 3.2A). Analysis of amino acid pools extracted from representative cells showed *E. coli pheT(G318W)* contained similar intracellular concentrations of *p*-Tyr and Phe as the wild type strain, indicating the *pheT* mutation has no effect on amino acid uptake (Table 3.1). In the absence of amino acid supplementation, the intracellular Phe:*p*-Tyr ratios were 1:1, and rose to 1:9 upon addition of *p*-Tyr. The growth of *E. coli pheT(G318W)* in the presence of *m*-Tyr, a non-proteinogenic amino acid previously shown to be a substrate for bacterial PheRS, was then investigated (258). Relative to wild type, growth of *E. coli* strain *pheT(G318W)* was inhibited in the presence of elevated intracellular concentrations of *m*-Tyr suggesting PheRS proofreading activity is needed to clear mischarged *m*-Tyr-tRNA^{Phe} *in vivo* (Table 3.1 and Fig. 3.2B). Editing assays performed *in vitro* confirmed that, as with *p*-Tyr, post-transfer editing of *m*-Tyr-tRNA^{Phe} by PheRS is ablated by the G318W mutation (Fig. 3.3). The inhibitory effect of *m*-Tyr on growth in the absence of editing was also

observed in *E. coli* mutants derived from strain MG1655 that, unlike the NP37 background, encodes an intact stringent response (Fig. 3.2C). Growth of *E. coli pheT(G318W)* was also evaluated in the presence of *ortho*-tyrosine (*o*-Tyr) and 3,4-dihydroxy-L-phenylalanine (L-DOPA), oxidation products of Phe and *p*-Tyr, respectively (279). Neither of these non-proteinogenic amino acids inhibited growth of wild type or the *pheT(G318W)* mutant *E. coli* strain (Fig. 3.4). The role of PheRS editing on yeast growth was tested under similar conditions to those examined for *E. coli*. While the editing deficient *frs1-1 (D243A)* yeast strain displayed no difference to wild type under heat shock or ethanol stress, it showed a pronounced defect in *p*-Tyr resistance. At elevated *p*-Tyr concentrations, growth of the *frs1-1 (D243A)* strain was restricted compared to wild type (Fig. 3.5A), while the growth of both strains was more comparably inhibited by addition of *m*-Tyr (Fig. 3.5B). These findings are in contrast to the responses of *E. coli* to tyrosine isomer stresses, consistent with the comparatively low Phe/*p*-Tyr amino acid specificity of the yeast enzyme and the previously observed inability of eukaryotic cytoplasmic PheRS to efficiently edit *m*-Tyr-tRNA^{Phe} (257, 258). The *frs1-1(D243A)* strain also exhibited a pronounced aging defect compared to wild type. Wild type entered stationary phase earlier and remained in this phase longer than the *frs1-1(D243A)* strain before a significant loss of viability was observed (Fig. 3.5C). By day 19, the *frs1-1(D243A)* strain showed approximately 100-fold lower survival rate than wild type. The significant decrease in lifespan of *frs1-1(D243A)* cells compared to wild type suggests that the ability to edit mischarged *p*-Tyr-tRNA^{Phe} by *Sc*ctPheRS is especially important to help maintain viability at low growth rates.

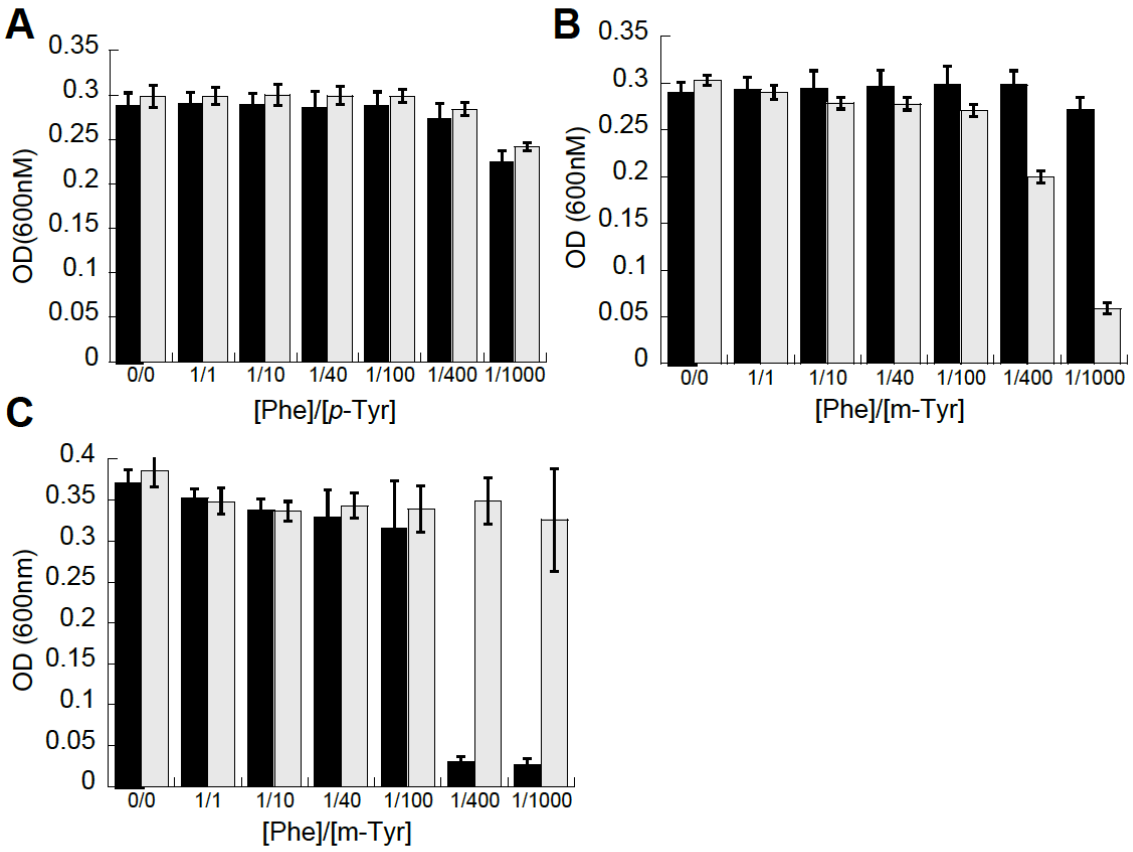


Figure 3.2. Effect of non-cognate amino acids on the growth of editing deficient *E. coli* strains. Growth of *E. coli pheT(G318W)* strain (grey bars) relative to wild type (black bars) under increasing concentrations of L-*p*-Tyr **(A)** or D,L-*m*-Tyr **(B)** relative to Phe. Cultures were grown in M9 minimal media supplemented with amino acids expressed as a ratio of Phe:Tyr. A ratio of 1:1 corresponds to 3 µM of each amino acid. **(C)** Growth of PheRS editing deficient strain of *E. coli* in an MG1655 background in the presence of different tyrosine isomers at 37°C. Bars are the mean of three independent cultures, with errors bars representing ± SD. Experiment performed by Dr. Tammy Bullwinkle.

Table 3.1. Amino acid pools in wild type and editing defective *E. coli* strains.

Strain	Supplement	*m*-Tyr (μM)[1]	*p*-Tyr (μM)	Phe (μM)	*p*-Tyr/Phe	*m*-Tyr/Phe
Wild type	+ *m*-Tyr	2.9±0.06	0.56±0.1	0.63±0.2	0.9±0.0	5±1
pheT(G318W)	+ *m*-Tyr	2.7±0.5	0.46±0.02	0.90±0.2	0.9±0.2	6±1
Wild type	+ *p*-Tyr	ND	11±4	0.91±0.1	12±4	ND[2]
pheT(G318W)	+ *p*-Tyr	ND	8.9±0.4	0.93±0.1	9.7±1	ND

[1]Concentrations of intracellular Phe and Tyr isomers isolated from wild type and *pheT(G318W) E. coli* strains grown in M9 minimal media supplemented with either *m*-Tyr or *p*-Tyr.
[2]ND indicates concentrations were below the detectable limit (0.01 μM).

Experiment performed by Dr. Tammy Bullwinkle, Dr. Michael Ibba and Dr. Kym Faull.

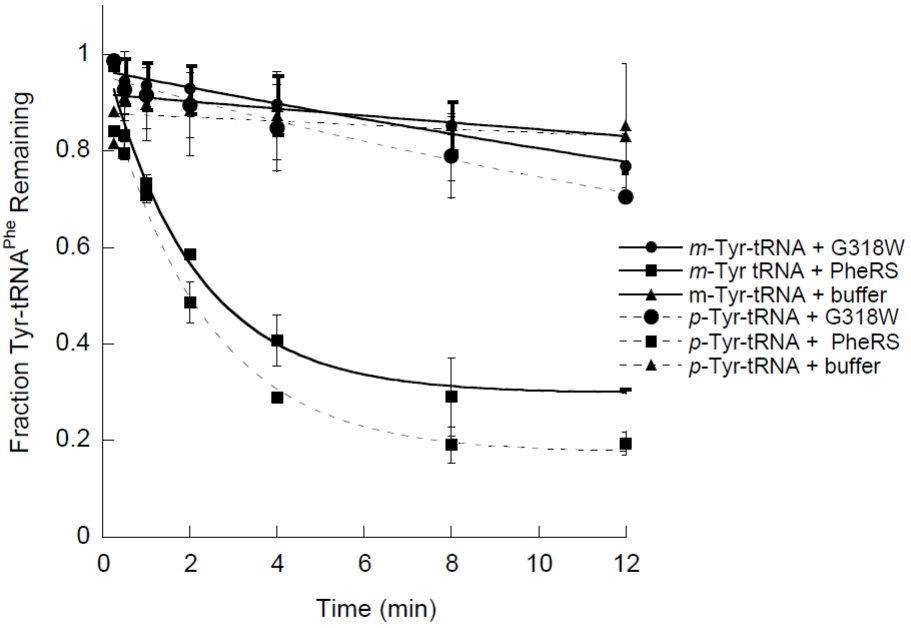


Figure 3.3. *Ec*PheRS post-transfer editing of mischarged tRNA^{Phe} substrates. Hydrolysis of 0.1 μM *E. coli p*-Tyr-[^{32}P]-tRNA^{Phe} (dashed lines) or *m*-Tyr-[^{32}P]-tRNA^{Phe} (solid lines) in the presence of 10 nM wild type *Ec*PheRS (■) G318W *Ec*PheRS (●) or buffer (▲) at 37^{o}C. Data points are the mean of three independent experiments, with errors bars representing ± SD. Experiment performed by Dr. Tammy Bullwinkle.

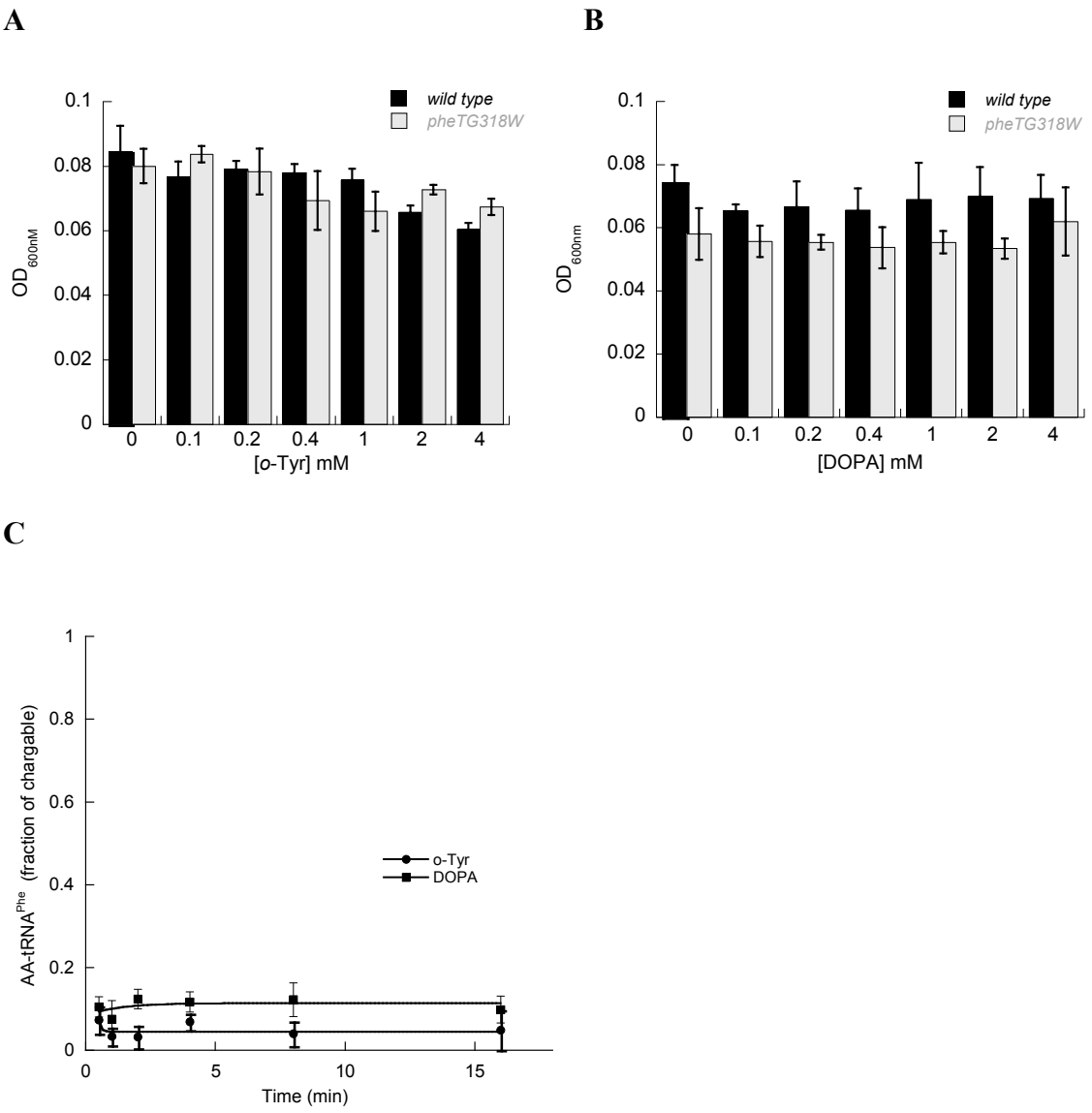


Figure 3.4. *E. coli* PheRS editing require requirement for tyrosine isomers. Growth of PheRS editing deficient *E. coli* at 37°C after 16 h in M9 minimal media supplemented with increasing concentrations of **(A)** *o*-Tyr or **(B)** L-dopa. **(C)** Aminoacylation of [^{32}P]-tRNA^{Phe} with *o*-Tyr (●) or L-dopa (■) by *E. coli* G318W PheRS (1μM). Bars are the mean of three independent cultures, with errors bars representing ± SD. Experiment performed by Dr. Tammy Bullwinkle and Eleftheria Matsa

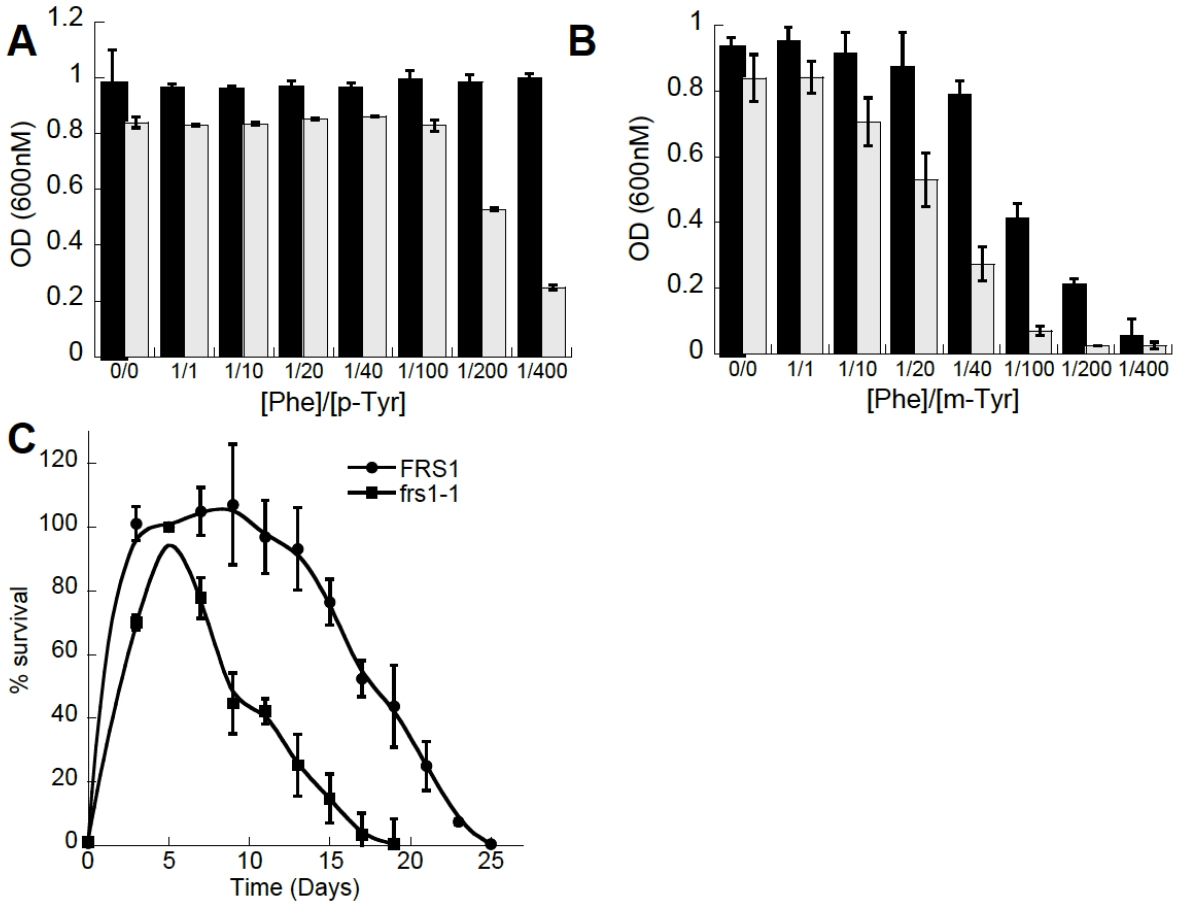


Figure 3.5. Effect of non-cognate amino acids on the growth of an editing deficient *S. cerevisiae* strain. Growth of yeast *frs1-1* (D243A) strain (grey bars) relative to a wild type strain (black bars) under increasing concentrations of L-*p*-Tyr **(A)** or D,L-*m*-Tyr **(B)** relative to Phe. Cultures were grown in minimal media supplemented with amino acids expressed as a ratio of Phe:Tyr. A ratio of 1:1 corresponds to 3 µM of each amino acid. **(C)** Chronological lifespan of *S. cerevisiae frs1-1* (D243A). Survival of wild type (NR1) and *frs1-1* (D243A) (NR2) cells grown in 50 mL of synthetic complete medium plus 0.003 mM Phe and a variable concentration of Tyr. Percent survival was normalized for each culture compared to CFU/mL at day 5. Data points are the mean of three independent cultures, with errors bars representing ±1 SD. Growth experiments performed by Medha Raina. Chronological lifespan of *S. cerevisiae frs1-1* (D243A) experiment performed by Dr. Noah Reynolds.

3.3.3 Loss of editing does not induce an ER stress response in *S. cerevisiae*

The growth defect observed for *Sc*ctPheRS editing deficient cells grown in the presence of excess *p*-Tyr suggested that under these conditions mistranslation might lead to up regulation of the unfolded protein response (UPR). To determine if the UPR is induced in *S. cerevisiae* cells lacking PheRS post-transfer editing activity, wild type and the *frs1-1(D243A)* strain were grown in either minimal media (MM) + Phe:*p*-Tyr 1:1 or MM + Phe:*p*-Tyr 1:50, and induction of the UPR was determined by quantifying splice variants of the UPR transcription activator, *HAC1* (280). Splice variants were only detected in cultures grown in the presence of tunicamycin, which inhibits glycosylation of newly synthesized proteins and induces the UPR. In minimal media, *HAC1* splicing is not induced in the PheRS editing deficient *frs1-1(D243A)* strain regardless of Phe/*p*-Tyr ratios (Fig. 3.6). This suggests that although editing deficient yeast cells can mis-translate Tyr for Phe and have a growth defect when grown under amino acid stress, this is not accompanied by mis-folded protein accumulation in the ER.

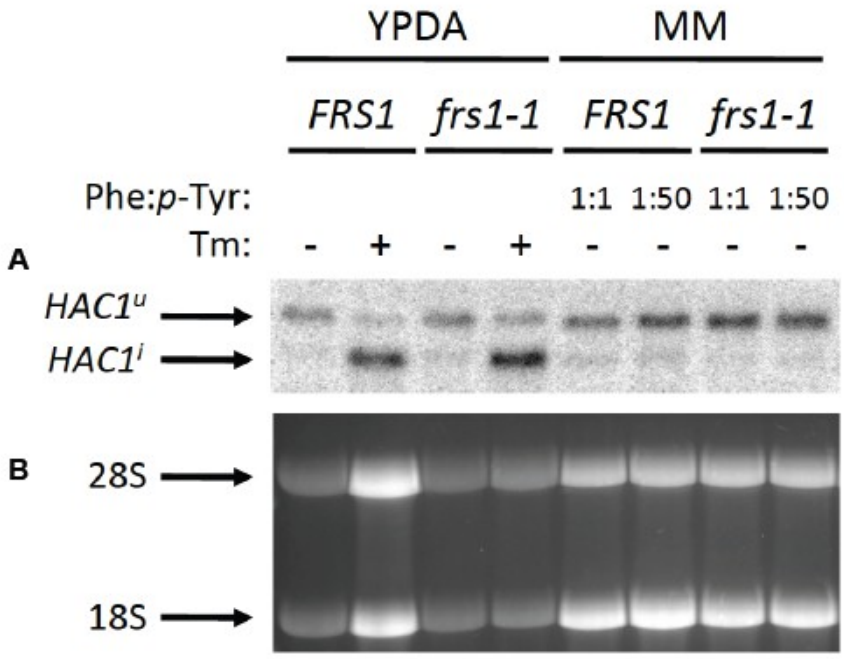


Figure 3.6. Loss of *S. cerevisiae* PheRS editing does not induce *HAC1* mRNA splicing. *FRS1* and *frs1-1* (D243A) cells were grown in YPDA, minimal media + 0.003 mM Phe, 0.003 mM *p*-Tyr (1:1), or minimal media + 0.003 mM Phe, 0.15 mM *p*-Tyr (1:50) 5 µg of total RNA was loaded onto a 1% agarose gel. The UPR was induced with 10 µg/mL tunicamycin (Tm). **(A)** Northern blot of *HAC1* mRNA splicing. Blot was probed for *HAC1* mRNA. The uninduced $HAC1^u$ and induced $HAC1^i$ splice variants of *HAC1* mRNA are indicated. **(B)** Agarose gel of total RNA with 28S and 18S ribosomal RNA transcripts indicated. Experiment performed by Dr. Noah Reynolds.

3.3.4 Bacterial and Eukaryotic PheRSs have divergent tyrosine isomer specificities

E. coli PheRS is able to edit preformed *m*-Tyr-$tRNA^{Phe}$ (258), and the loss of this activity in the G318W variant indicates that editing occurs at the site previously described for *p*-Tyr-$tRNA^{Phe}$ [(277), Fig. 3.3]. Wild type *Ec*PheRS did not stably charge $tRNA^{Phe}$ with either *m*- or *p*-Tyr, while G318W utilized both isomers for aminoacylation, with *m*-tyr

being a more efficient substrate (Figs. 3.7A and B). Under similar conditions, G318W PheRS was unable to utilize *o*-Tyr or L-DOPA for $tRNA^{Phe}$ aminoacylation, consistent with the absence of any growth phenotype of the *pheT(G318W)* strain in the presence of these tyrosine analogs (Fig. 3.4). As a substrate for *T. thermophilus* PheRS, L-DOPA has been shown to be 1500-fold less efficient than Phe (281). Examination of amino acid substrate specificity showed the catalytic efficiency (k_{cat}/K_M) for *m*-Tyr activation by *Ec*PheRS to be 35-fold less than for Phe, in contrast to *p*-Tyr which is activated almost 3000-fold less efficiently than the cognate substrate (Table 3.2). The ability of *Ec*PheRS to efficiently activate *m*-Tyr is consistent with the need for editing to maintain cellular viability during growth in the presence of this non-proteinogenic amino acid.

In contrast to the *E. coli* enzyme, wild type *Sc*ctPheRS efficiently utilizes *m*-Tyr for activation and aminoacylation of $tRNA^{Phe}$. Charging of $tRNA^{Phe}$ with *m*-Tyr was seen at amino acid substrate concentrations where *p*-Tyr-$tRNA^{Phe}$ synthesis was not detected (Fig. 3.7C, Table 3.2). The k_{cat}/K_M of *m*-Tyr activation by *Sc*ctPheRS is 71-fold lower than that of Phe, demonstrating relatively poor discrimination between the two amino acids (Table 3.2). In contrast to the *E. coli* enzyme, *p*-Tyr-$tRNA^{Phe}$ is a better substrate for post-transfer editing by *Sc*ctPheRS relative to *m*-Tyr-$tRNA^{Phe}$ (Fig. 3.8). These results provide a possible explanation for the toxic effects *m*-Tyr has on the wild type yeast strain (Fig. 3.5B), although additional cytotoxic affects of *m*-Tyr outside of translation cannot be ruled out. o-Tyr and L-DOPA also had similar toxic effects on the wild type like *m*-Tyr indicating that these isomers are not good substrates for *Sc*ctPheRS (Fig. 3.9) . Post-transfer editing of *m*-Tyr-$tRNA^{Phe}$ by *Sc*ctPheRS provides some

protection from *m*-Tyr's toxic affects as there is a difference in the growth of wild type versus the *frs1-1(D243A)* strain at high concentrations of *m*-Tyr (Fig 3.5B). The mitochondrial variant of yeast PheRS (*Sc*mtPheRS), which naturally lacks Tyr-tRNA^{Phe} post-transfer editing activity (263), was also found to synthesize *m*-Tyr-tRNA^{Phe} more efficiently than *p*-Tyr-tRNA^{Phe} at similar tyrosine isomer concentrations (Fig. 3.7D). The absence in yeast of appropriate quality control pathways in either the cytoplasm or mitochondria suggests that *m*-Tyr toxicity results from the accumulation of mischarged tRNAs in both compartments.

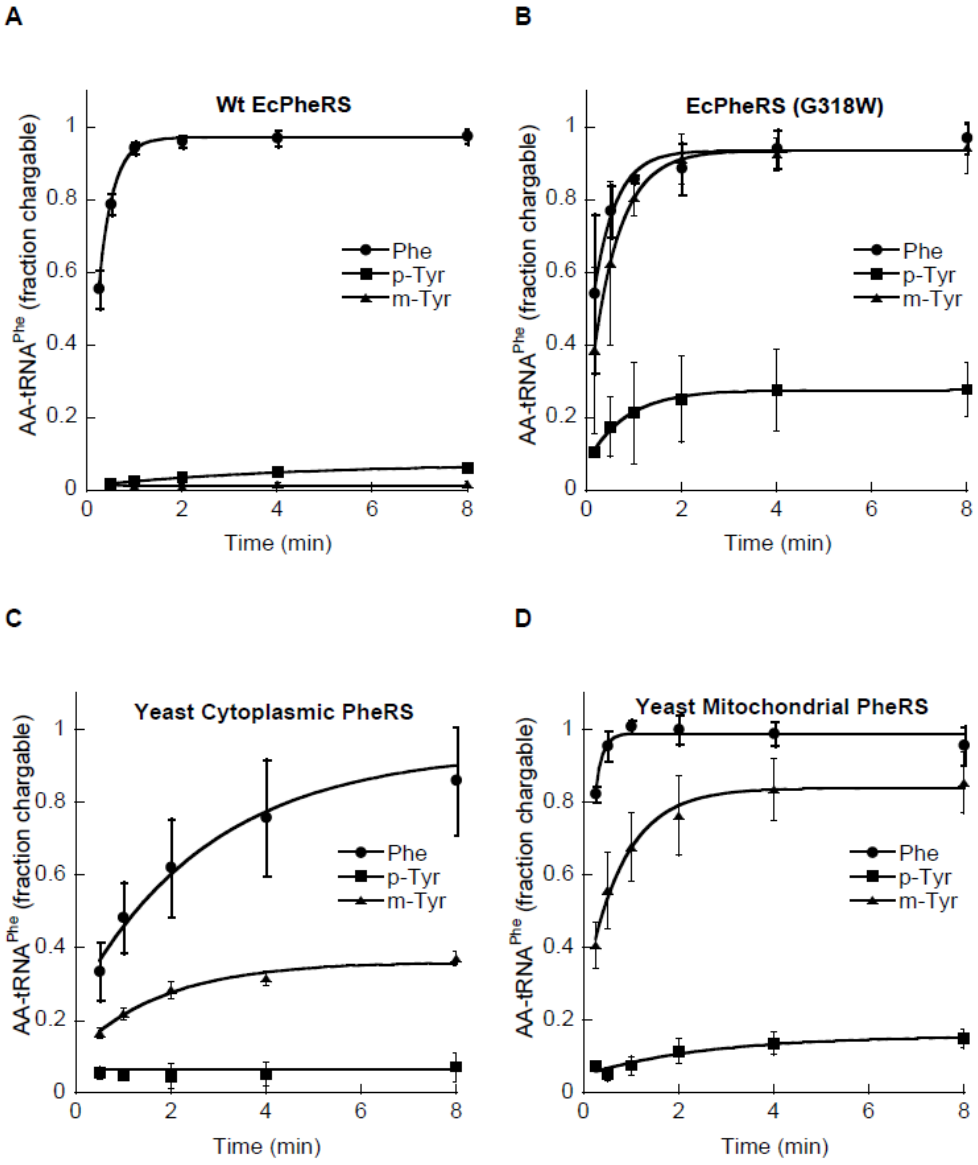


Figure 3.7. Tyrosine isomers as substrates for tRNA^{Phe} aminoacylation by PheRS variants. tRNA^{Phe} aminoacylation activities of **(A)** wild type and **(B)** G318W *E. coli* PheRS for 60 μM cognate Phe and non-cognate *p*- and *m*-Tyr substrates. Aminoacylation activities of **(C)** wild type cytoplasmic and **(D)** wild type mitochondrial *S. cerevisiae* PheRS for 100 μM cognate Phe and non-cognate *p*- and *m*-Tyr substrates. Data points are the mean of three independent experiments, with errors bars representing ± SD. Experiment performed with Dr. Tammy Bullwinkle.

Table 3.2. Steady-state kinetic constants for amino acid activation by PheRS from *E. coli* and *S. cerevisiae* cytoplasmic PheRS.

PheRS	Phe			*m*-Tyr			*p*-Tyr	Specificity ($k_{cat}/K_M/k_{cat}/K_M$)	
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}/μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}/μM)	k_{cat}/K_M (s^{-1}/μM)	Phe/*m*-Tyr	Phe/*p*-Tyr
E. coli	18±4	5.2±2	0.29	247±60	2.1±0.8	0.008	1.1x10-4	35	2650
Yeast ct	16±2	26±4	1.6	1150±230	26±4	0.023	0.014	71	120

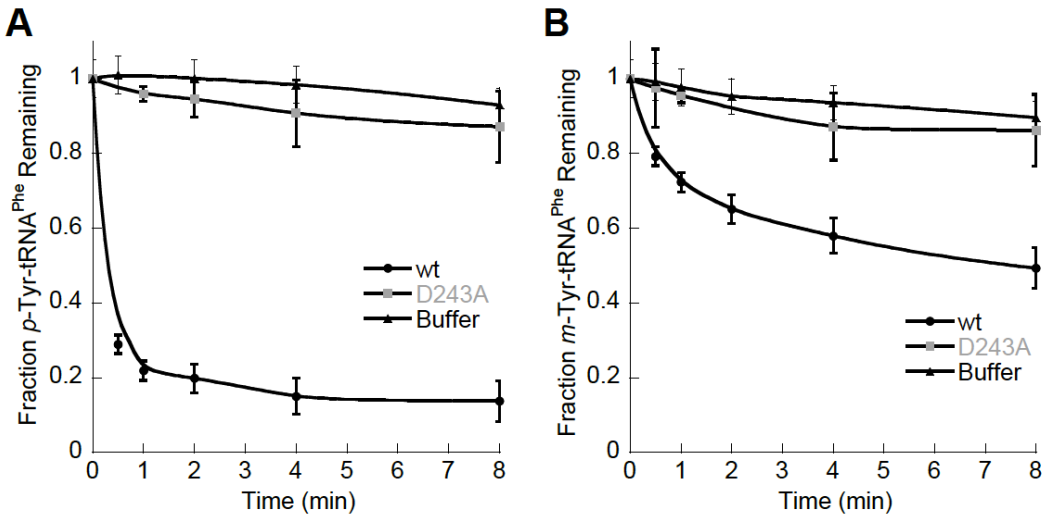


Figure 3.8. *Sc*ctPheRS post-transfer editing of mischarged $tRNA^{Phe}$ substrates. Hydrolysis of 0.1 μM yeast **(A)** *p*-Tyr-[^{32}P]-$tRNA^{Phe}$ or **(B)** *m*-Tyr-[^{32}P]-$tRNA^{Phe}$ in the presence of 10 nM wild type *Sc*ctPheRS (●) D243A *Sc*ctPheRS (■) or buffer (▲) at 37°C. Data points are the mean of three independent experiments, with errors bars representing ± SD. Experiments performed with Dr. Tammy Bullwinkle.

A **B**

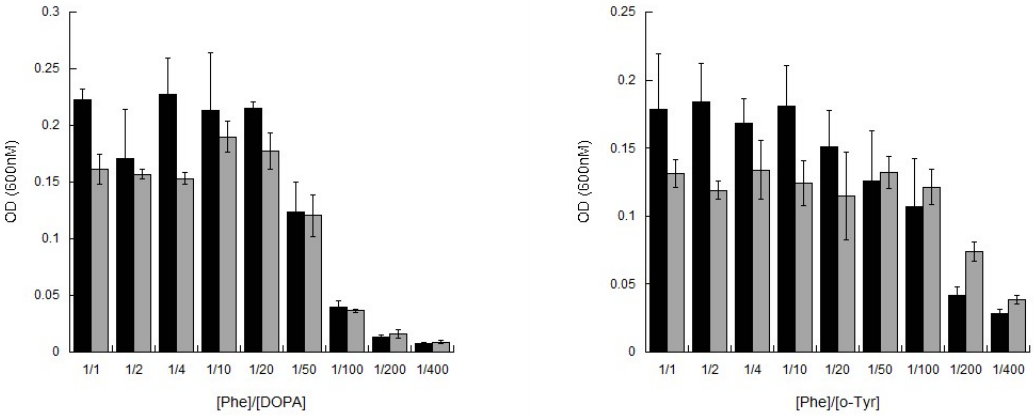


Figure 3.9. *Sc*ctPheRS editing requirement for tyrosine isomers. Growth of yeast *frs1-1* (D243A) strain (grey bars) relative to a wild type strain (black bars) under increasing concentrations of L-DOPA **(A)** or o-Tyr **(B)** relative to Phe. Cultures were grown in minimal media supplemented with amino acids expressed as a ratio of Phe:Tyr isomer. A ratio of 1:1 corresponds to 3 μM of each amino acid.

3.3.5. *m*-Tyr is incorporated into the *E. coli* proteome at Phe Codons

The correlation between *E. coli* PheRS-dependent *m*-Tyr toxicity *in vivo* and synthesis of *m*-Tyr-tRNA^{Phe} *in vitro* strongly suggests that this mischarged tRNA is a substrate for ribosomal peptide synthesis. Dipeptide synthesis was monitored *in vitro* using *m*-Tyr-tRNA^{Phe}**:**EF-Tu:GTP as a substrate for decoding of a ribosomal A site Phe (UUC) codon. Under these conditions similar levels of fMet-*m*-Tyr and fMet-Phe were synthesized, indicating a lack of discrimination against the non-proteinogenic amino acid at the A-site of *E. coli* ribosomes (Fig. 3.10A).

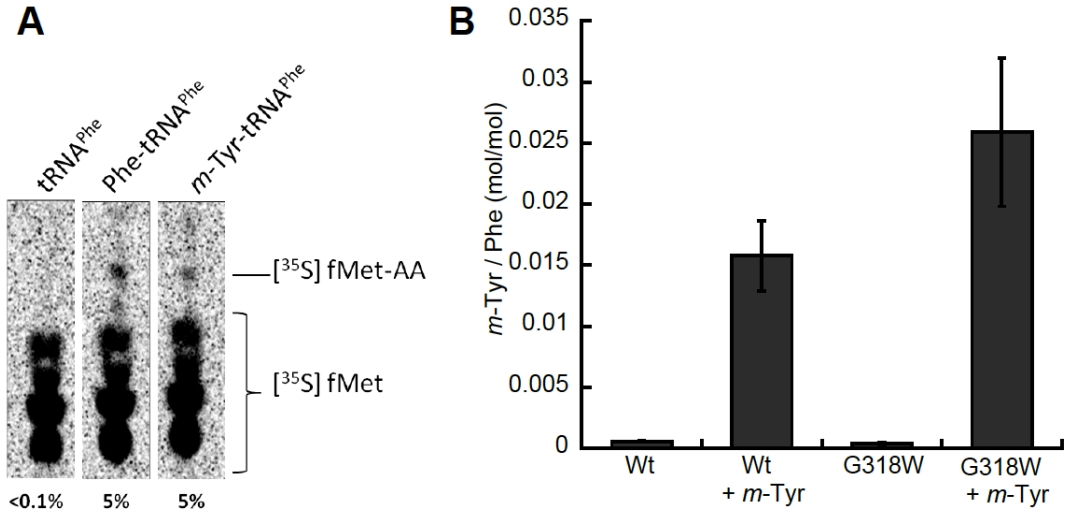


Figure 3.10. Incorporation of m-Tyr into the proteome of *E. coli*. (A) *In vitro* 70S ribosomal di-peptide synthesis with either Phe-tRNA^{Phe} or *m*-Tyr-tRNA^{Phe} **(B)** LC-MS/MS-MRM quantification of *m*-Tyr and Phe in protein hydrolysis isolated from *E. coli* expressed as molar ratio of *m*-Tyr to Phe. Wild type (Wt) and *pheT*(G318W) strains grown in M9 minimal media alone and supplemented with *m*-Tyr are shown. Error bars represent ± standard error of means. Experiment performed by Dr. Tammy Bullwinkle.

The effect of *m*-Tyr on protein synthesis *in vivo* was investigated by analyzing the accumulation of the non-proteinogenic amino acid in the proteomes of wild type and *E. coli pheT(G318W)* cells. Cytosolic protein samples were isolated from *m*-Tyr treated *E. coli* cells and samples subjected to acid hydrolysis to generate individual amino acids. The resulting amino acid hydrolysate was analyzed by liquid chromatography tandem mass spectrometry with multiple reaction monitoring (LC-MS/MS-MRM). To validate peak assignments of the Tyr isomers, co-chromatography was performed with synthetic *m*-Tyr or *o*-Tyr (*o*-Tyr) added to proteome samples. Only one peak for each of the isomers was observed, validating the assignments. Comparison of proteome total amino

acid levels between wild type and *pheT*(G318W) strains indicated a level of misincorporation of 1 % *m*-Tyr at Phe codons due to the absence of PheRS editing (Fig. 3.10B). In wild type proteins the fraction of *m*-Tyr compared to Phe is 0.015, increasing to 0.025 in samples isolated from the *pheT(G318W)* strain grown in the same conditions. This result indicates post-transfer editing by PheRS provides protection of the *E. coli* proteome from misincorporation of *m*-Tyr at Phe codons. Quantification of *p*-Tyr relative to Phe in the protein samples isolated from cultures grown in the presence of 0.5 mM *p*-Tyr does not change between the wild type and *pheT(G318W)* strains indicating this protein amino acid is not significantly misincorporated at Phe codons, even in the absence of PheRS editing (Fig. 3.11). These analyses show a ratio of *p*-Tyr/Phe of 0.6, which correlates reasonably well with previous estimates of amino acid usage in *E. coli* [0.7, (282)].

A detectable level of *m*-Tyr in the proteome of wild type *E. coli* suggests either this non-proteinogenic amino acid escapes PheRS editing, infiltrates the proteome by means other than misincorporation at Phe codons or is carried over during cytosolic protein preparation. To measure the approximate amount of carryover, wild type PheRS *E. coli* strain was grown in the presence of 0.5 mM *o*-Tyr, which is not a substrate for protein synthesis, and total protein samples were subjected to acid hydrolysis and LC-MS/MS-MRM. In these samples, traces of *o*-Tyr were detected, indicating that free amino acid carry over possibly contributes to some of the *m*-Tyr detected in the samples from the wild type strain grown in M9 minimal media supplemented with *m*-Tyr. Whether the *m*-Tyr seen in the proteome of *E. coli* containing PheRS editing is formed

post-translationally or is incorporated during protein synthesis via another promiscuous tRNA synthetase in *E. coli* is unclear. Aminoacylation of $tRNA^{Tyr}$ with *m*-Tyr by *E. coli* TyrRS was detected *in vitro*, suggesting this synthetase may provide a route of *m*-Tyr incorporation even when PheRS editing is active (Fig. 3.12).

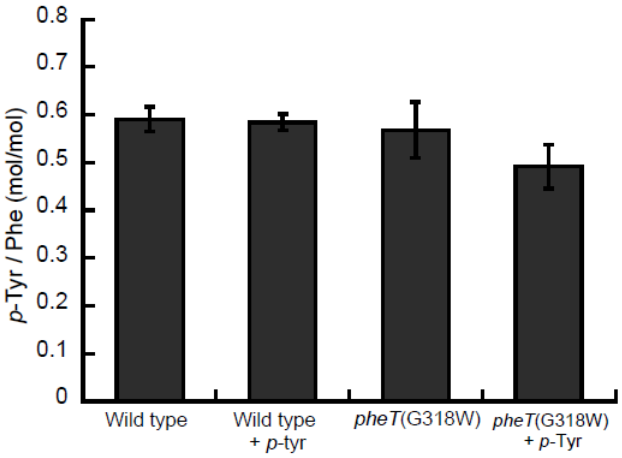


Figure 3.11. *p*-Tyr is not misincorporated in the proteome of *E. coli* at Phe codons. Mass spectroscopy quantification of *p*-Tyr and Phe in protein hydrolysis isolated from *E. coli* expressed as molar ratio of *p*-tyr to Phe. Wild type and *pheT*(G318W) strains grown in in M9 minimal media alone and supplemented with *p*-Tyr are shown. Error bars represent ± standard error of means. Experiment performed by Dr. Tammy Bullwinkle and Dr. Kym Faull.

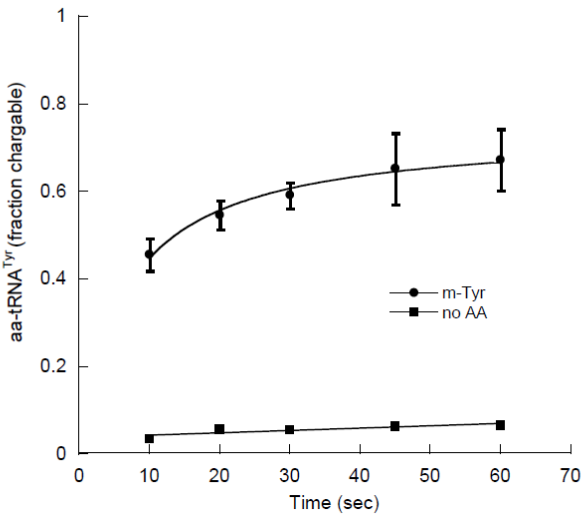


Figure 3.12. *E. coli* TyrRS uses *m*-Tyr. Aminoacylation of *E. coli* [^{32}P]-tRNA^{Phe} transcript (0.5 μM) with *m*-Tyr (1 mM) by *E. coli* TyrRS (50 nM) at 25 ^{o}C. Experiment performed with Dr. Tammy Bullwinkle.

3.3.6 *E. coli* PheRS editing is required for growth under oxidative stress conditions

Reactive oxygen species (ROS) generated under oxidative stress via the Fenton reaction are capable of catalyzing the conversion of Phe to *m*-Tyr, which could potentially threaten the fidelity of protein synthesis in the absence of editing (260, 279). To investigate if oxidative stress conditions generate potentially toxic levels of *m*-Tyr *in vivo*, wild type and editing deficient *E. coli* were grown in the presence of H_2O_2 and $FeSO_4$ (Fe^{2+}) as a source of ROS. LC-MS/MS-MRM analyses showed that *m*-Tyr accumulated in the intracellular amino acid pools of ROS-treated cells (Fig. 3.13A). In addition to *m*-Tyr, significant *de novo o*-Tyr accumulation was also observed following

ROS treatment, although this is not expected to pose a threat to translation fidelity as it is not a substrate for PheRS (Fig. 3.4, Figure 3.13A). *E. coli* lacking PheRS editing activity showed a reduction in growth relative to wild type when grown in media where ROS exposure increased, consistent with the accumulation of free *m*-Tyr and its subsequent utilization in protein synthesis (Fig. 3.13B). Taken together, our data indicate that PheRS editing activity affords *E. coli* protection against the co-translational insertion of non-proteinogenic amino acids that accumulate during oxidative stress.

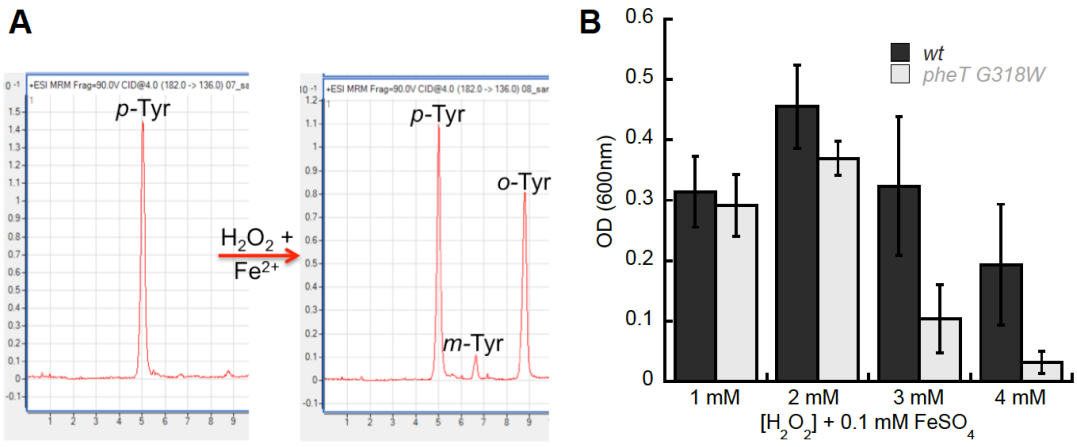


Figure 3.13. Requirement for PheRS post-transfer editing in ROS conditions *in vivo*. (**A**) LC-MS/MS-MRM chromatograms for *p*-, *m*- and *o*-Tyr (m/z 182→136 transition) extracted from cells grown in the absence (left) and presence (right) of H_2O_2 and $FeSO_4$. (**B**) Growth of *E. coli pheT(G318W)* strain relative to wild type in M9 minimal media supplemented with 0.1 mM $FeSO_4$ and increasing concentrations of H_2O_2. Bars are the mean of three independent cultures, with errors bars representing ± SD. LC-MS/MS experiment done by Dr. Kym Faull and *E. coli* growth assay with H_2O_2 done by Dr. Tammy Bullwinkle.

3.4 Discussion

3.4.1 Context dependent specificity and editing

It has long been proposed that in order to prevent elevated levels of mistranslation, the fidelity of aminoacyl-tRNA synthetases needs to be at or above 1 in 3,000, which is cited as an approximate overall level of error for protein synthesis (8). AaRS fidelity is achieved through discrimination at the aminoacylation site as well as through additional editing activities in some aaRSs. Protection against both *p*-Tyr and *m*-Tyr incorporation at Phe codons appears critical in *E. coli* as the PheRS enzyme maintains high active-site selectivity against *p*-Tyr as well as post-transfer editing activity against *m*-Tyr-tRNA^{Phe}. *E. coli* PheRS requires this editing activity to protect the proteome from toxic effects of the non-proteinogenic amino acid *m*-Tyr, which is poorly discriminated against by the active site of the enzyme. Examination of the structure of the catalytic active site provides clues as to why PheRS is unable to discriminate against all the Tyr isomers. Ala294 is primarily responsible for specificity against binding of *para*-substituted Phe analogs, while Gln174 and Glu210 help stabilize the hydroxyl of the non-cognate *m*-Tyr at position 3 of the ring (*E. coli* numbering) (258). In the case of the cognate Phe substrate, Glu210 is also needed to hydrogen bond with the Phe amino group, ensuring correct orientation of the substrate for activation (283, 284). It is unlikely this enzyme selects against recognition of *m*-Tyr while still maintaining efficient activity for the cognate amino acid; therefore, the maintenance of post-transfer editing activity is critical for fidelity in *E. coli*. In eukaryotes, cytoplasmic PheRS editing is needed to protect the proteome from *p*-Tyr misincorporation. This finding concurs with the low Phe/*p*-Tyr

specificity of the yeast cytoplasmic enzyme (257). It is unclear if protection from *m*-Tyr incorporation is achieved through editing as the yeast strain encoding wild-type ctPheRS is sensitive to high concentrations of *m*-Tyr, mtPheRS efficiently aminoacylates *m*-Tyr onto $tRNA^{Phe}$, and other eukaryotic proteomes are vulnerable to the use of this oxygen-damaged amino acid in translation (261). Taken together, these findings suggest that either *m*-Tyr accumulation is not a substantial threat in eukaryotes, or possibly that the incorporation of low amounts of this non-proteinogenic amino acid in certain proteomes confers some as yet unknown evolutionary benefits.

3.4.2 Non-proteinogenic amino acids as threats to translational integrity

Naturally occurring non-proteinogenic amino acids occur widely in nature and are well-characterized by-products and/or intermediates of biosynthetic pathways (285). The actual threats these non-canonical substrates pose to protein synthesis and cell viability is unknown, as is the role of aaRS quality control in protecting the proteome from such amino acids. The non-proteinogenic amino acid *m*-Tyr has been detected in several eukaryotic proteomes and is one of the products of canonical aromatic amino acid oxidation (286, 287). The presence of hydroxylated forms of tyrosine in proteomes has previously been attributed to post-translational damage to proteins by hydroxyl radical species, and is often used as a marker for tissue damage due to the oxidative conditions of aging and disease. It has also been shown that *m*-Tyr and other Tyr analogues, for example L-DOPA, are substrates for translation in some organisms and could potentially be incorporated directly during protein synthesis (258, 261, 281, 288, 289). Our results

now reveal the role of *E. coli* PheRS editing for preventing the use of *m*-Tyr during protein synthesis, demonstrating the threat amino acid oxidation poses to the proper functioning of the bacterial translation machinery.

Incorporation of *m*-Tyr into the proteome of *E. coli* at Phe codons is toxic to the cell, and in the absence of PheRS quality control this non-proteinogenic amino acid serves as an efficient substrate for translation. Other non-proteinogenic amino acids have also been shown to be potential threats to translation, such as α-aminobutyrate, which in the absence of ValRS editing is toxic at high concentrations (290). The robust editing activity maintained by *E. coli* PheRS to protect the proteome from *m*-Tyr demonstrates the significant threat such an amino acid poses when misincorporated at specific near-cognate positions. In contrast, the presence of *m*-Tyr in the proteome of wild-type *E. coli* suggests misincorporation also occurs at Tyr codons but without cytotoxic sequelae, indicating that the effects of non-proteinogenic amino acid incorporation are context-dependent. The replacement of *p*-Tyr with this non-proteinogenic amino acid appears undisruptive to cell viability, whereas PheRS editing is crucial for preventing toxic effects of *m*-Tyr translated at Phe codons. The cell does not have codons or tRNAs for *m*-Tyr, therefore any advantage or disadvantage this amino acid might provide to the proteome cannot easily be selected for, or against, at the level of the genetic code. The only selection against near-cognate non-proteinogenic amino acid use during translation can be made at the level of the synthetase or ternary complex formation with an elongation factor (291). In *E. coli*, misincorporation of *m*-Tyr at Phe codons in the absence of PheRS quality control occurred at a frequency of 1 % and had a significant

impact on cellular viability and restricted growth. This contrasts with the effects of misincorporation of canonical amino acids, which have been shown to be tolerated at rates of up to 10 % without inhibiting growth (292). Taken together with earlier studies, our present findings now show that the misincorporation of non-proteinogenic amino acids presents a far greater challenge for protein synthesis quality control than does canonical mistranslation. This in turn suggests that many "dispensable" editing functions, both in aaRSs and *trans* editing factors, may actually be essential for growth under conditions that lead to the accumulation of potentially toxic levels of non-proteinogenic amino acids.

3.4.3. Oxidative stress and translation quality control

Oxidation of amino acids by reactive species such as hydroxyl radical and superoxide anions results in limited alteration of amino acid structure, such as the addition of a hydroxyl group, creating potential *in vivo* substrates for tRNA misacylation. These damaged amino acids challenge the protein synthesis machinery, as for example in the case of L-DOPA, and leucine hydroxide, which have been shown to be incorporated into proteins in mouse cells (288, 289). The formation of intracellular *m*-Tyr in *E. coli* under physiological conditions is possibly a result of cellular exposure to H_2O_2. Aerobic respiration results in elevated endogenous levels of H_2O_2, but bacterial cells are also exposed to ROS present in their environment. Uncharged H_2O_2 is able to penetrate the cell membrane and accumulate inside cells whenever H_2O_2 is present in the extracellular habitat. At physiological pH, H_2O_2 quickly oxidizes ferrous iron via the Fenton reaction,

generating a hydroxyl radical that can react with nearby cellular targets (293). Accumulation of toxic levels of *m*-Tyr in the intracellular pools of *E. coli* occurs under experimental conditions that promote the formation of hydroxyl radicals; however, this is not the major byproduct of Phe oxidation. The *o*-Tyr isomer is the more abundant hydroxylation product under ROS-generating conditions used here (Fig. 3.13). However, there is no observed *o*-Tyr aminoacylation of $tRNA^{Phe}$ by wild-type PheRS *in vitro*, or inhibition of cell growth in the presence of this hydroxylated Phe substrate. These, and the corresponding biochemical data, indicate how *E. coli* PheRS has evolved to effectively discriminate against different Tyr isomers using a combination of substrate specificity (*o*-Tyr, *p*-Tyr) and editing activity (*m*-Tyr, *p*-Tyr). In contrast, yeast PheRS has mainly evolved specifically to discriminate for *p*-Tyr by editing, reflecting differences in the factors that drive selection of quality control mechanisms.

Chapter 4:

Reduced amino acid specificity of mammalian Tyrosyl-tRNA synthetase is associated with elevated mistranslation of Tyr codons

4.1 Introduction

Translation accuracy is vital for the maintenance of cellular integrity. Accuracy in protein synthesis is dependent on a combination of sequential substrate recognition events, which include the synthesis of correct aminoacyl tRNAs (aa-tRNA) by aminoacyl tRNA synthetases (aaRS), binding of elongation factor 1A (EF1A) to the cognate aa-tRNA and the selection of the correct aa-tRNA by the ribosome. All these steps have their own inherent error rate which is thought to vary depending on various environmental conditions. The overall error rate of translation is generally believed to be 10^{-4} (8, 9), with the first step, synthesis of aa-tRNA, being the most error prone. Aa-tRNA synthesis is a two step reaction: activation of an amino acid with ATP to form aminoacyl adenylate, followed by transfer of the aminoacyl moiety to the 3' end of the tRNA, (3) . The error rate of this first step of translation is largely dependent on the specificity of the aaRS,

which is selection of the correct amino acid and tRNA from the respective cellular pools of predominantly non-cognate substrates. AaRSs select their cognate tRNAs by exploiting sequence specific differences between various tRNAs during binding and aminoacylation thereby resulting in a low error rate of 10^{-6} at this step (6, 10). In contrast, selection of the correct amino acid is often challenging due to the lack of sufficient discriminating functional groups in many amino acids and their analogs. In order to maintain a comparatively low error rate during translation, editing mechanisms have evolved to discriminate between substrates with close structural and chemical properties by hydrolyzing either the activated non-cognate amino acid (pre-transfer editing) or mischarged tRNA (post-transfer editing) (20). The high specificity displayed by some aaRSs is also achieved by taking advantage of the unique structural and chemical properties of certain amino acids, leading to favorable binding affinities of cognate over non-cognate substrates in the active site of the enzyme. For example, Phe and Tyr differ from each other by a single hydroxyl group, the specific recognition and binding of which allows bacterial tyrosyl-tRNA synthetase (TyrRS) to discriminate against non-cognate Phe with a specificity of 10^5 (294).

Although quality control at different steps can limit errors to approximately one mistranslated codon per 10,000 during mRNA-directed protein synthesis (8, 9, 295), recent studies suggest that error rates vary considerably during translation. In *Escherichia coli* codon specific differences in error rates of up to 18-fold were observed using a luciferase reporter assay (9). More dramatically, exposure of mammalian cells to a variety of stresses elevates tRNA mischarging to levels that could potentially lead to

increases in the error rate of translation of 100-fold or more for some codons (52, 296). Recent studies showed misincorporation rates of up to 0.7% for Phe at Tyr codons during protein synthesis in CHO cells under conditions of amino acid depletion (297, 298). Here we investigate the cause of mistranslation observed in CHO cells. Also, mutation of the CHO active site residues were carried out to help determine residues important for substrate recognition and discrimination in the eukaryotic TyrRS compared to the bacterial enzyme.

4.2 Materials and methods

4.2.1 Cell culture experiment setup and analytics

CHO cells producing a recombinant monoclonal antibody were grown in chemically defined media. Tyr $2Na^{+}\cdot 2H_2O$ (SAFC Biosciences, Lenexa, KS) was used in the supplementation study. All media and stock solutions were filter-sterilized at 0.1 μm. Cells were grown in 500 mL vented shake flasks under 36 °C, 5% CO_2 and 160 rpm. The inoculation density was $1x10^6$ cells/mL and the culture was grown for 16 days. Bolus feeds were added on days 5, 7, 9, 11, and 13 at 9 % of current working volume. Tyr supplement was added on days 9, 11, and 13 targeting a 1 mM addition to the culture. Glucose (Life Technology, Carlsbad, CA) was maintained in the range of 6 – 8 g/L throughout production. Viable cell density (VCD) and viability were measured by CEDEX (Innovatis, Germany) and metabolites by NOVA BioProfile (NOVA Biomedical, MA). Values of pH, pO_2, and pCO_2 were analyzed by the Bioprofile pHox

(NOVA Biomedical, MA) and osmolality by the model 2020 osmometer (Advanced Instruments, Norwood, MA). Titer was measured by reverse-phase HPLC (Waters, Milford, MA) using a Protein A column (Life Technologies, Carlsbad, CA). Free amino acids were measured by cation exchange HPLC (Agilent Technologies, Santa Clara, CA).

4.2.2 Cloning and mutagenesis

The CHO TyrRS (EGW00102) gene, codon optimized for expression in *E. coli*, was synthesized (GenScript) and subcloned under T7 promoter control into pET33b vector at NcoI and XhoI restriction sites. The resulting plasmid pET33b-TyrRS-His_6 was used to transform *E. coli* BL21(DE3) cells. CHO TyrRS mutations were constructed by PCR amplification and Dpn1 digestion using primers listed in Table 4.1. All cloning and mutagenesis were confirmed by sequencing and the resulting plasmids used to transform *E. coli* BL21 XJB (DE3).

Table 4.1. Primers used for CHO TyrRS mutagenesis.

Primer Name	Sequence	Position of residue
CHOHis77Thr_F	5' CCTGTTCGCGGATCTGacCGCTTATCTGGACAAC 3'	His77
CHOHis77Thr_R	5' GTTGTCCAGATAAGCGgtCAGATCCGCGAACAGG 3'	His77
CHOA74G_F	5' GTGACCATCCTGTTCGgcGATCTGCACGCTTATC 3'	Ala74
CHOA74G_R	5' GATAAGCGTGCAGATCgcCGAACAGGATGGTCAC 3'	Ala74
CHON82D_F	5' GATCTGCACGCTTATCTGGACgatATGAAAGCGCCGTGGGAACTG 3'	Asn82
CHON82D_R	5' CAGTTCCCACGGCGCTTTCATatcGTCCAGATAAGCGTGCAGATC 3'	Asn82
CHOG120N_F	5' CTGAAATTTATCAAAaaCACCGACTACCAGCTGTC 3'	Gly120
CHOG120N_R	5' GACAGCTGGTAGTCGGTGttTTTGATAAATTTCAG 3'	Gly120
CHOY123W_F	5' GAAATTTATCAAAGGCACCGACTggCAGCTGTCCAAAGAATATACGC 3'	Tyr123
CHOY123W_R	5' GCGTATATTCTTTGGACAGCTGccAGTCGGTGCCTTTGATAAATTTC 3'	Tyr123
CHOW40C_F	5' GAACTGAAAGTTTATTGcGGCACCGCGACCACGGG 3'	Trp40
CHOW40C_R	5' CCCGTGGTCGCGGTGCCgCAATAAACTTTCAGTTC 3'	Trp40
CHOY52H_F	5' CGGGTAAACCGCATGTTGCCcatTTCGTCCCGATGTC 3'	Tyr52
CHOY52H_R	5' GACATCGGGACGAAatgGGCAACATGCGGTTTACCCG 3'	Tyr52
CHOD122N_F	5' GAAATTTATCAAAGGCACCaACTACCAGCTGTCCAAAG 3'	Asp122
CHOD122N_R	5' CTTTGGACAGCTGGTAGTtGGTGCCTTTGATAAATTTC 3'	Asp122
CHOL125W_F	5' CAAAGGCACCGACTACCAGtgGTCCAAAGAATATACGCTG 3'	Leu125
CHOL125W_R	5' CAGCGTATATTCTTTGGACcaCTGGTAGTCGGTGCCTTTG 3'	Leu125

4.2.3 Purification of CHO TyrRS and variant proteins

Protein was produced by growing the cells to an optical density at 600 nm (OD_{600}) of 0.6 at 37 °C, 250 r.p.m. Gene expression was induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 hrs. Cells were harvested; the pellet was resuspended in a buffer containing 25 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol and 5 mM imidazole, and flash frozen using liquid N_2 before storage at −80 °C. Cell-free extract was produced by sonication of cells in buffer A (25 mM Tris–HCl, pH 8.0, 300 mM NaCl, 5 mM imidazole and 10% glycerol) containing a protease inhibitor mixture tablet (Complete Mini, EDTA-free; Roche Applied Science) followed by centrifugation at 150,000×*g* for 45 min. The resulting supernatant was loaded onto a pre-equilibrated 3 ml TALON® resin metal affinity column (Clontech) followed by washing, and the protein was eluted with Buffer B (25 mM Tris–HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole and 5% glycerol). Fractions containing the protein of interest (judged by Coomassie Brilliant Blue staining after SDS–PAGE) were pooled and dialyzed twice against Buffer C (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol and 5 % Glycerol) to remove any bound tyrosyl-adenylate from TyrRS. The enzyme was further dialyzed against two buffer changes of Buffer D (50 mM Tris-HCl, pH 7.5, 140 mM KCl, 20 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 5 % glycerol) and finally against Buffer D with 50% glycerol and stored at -20 °C. Mini-TyrRS was produced as described for CHO TyrRS except that the region of pET33b-TyrRS-His_6 encoding the EMAP-II like domain was removed.

4.2.4 Cloning and in vitro transcription of CHO $tRNA^{Tyr}$

The gene for CHO $tRNA^{Tyr}_{GTA}$ (CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT GTAGATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGACCA) was chosen from the various $tRNA^{Tyr}$ gene sequences predicted by tRNAscan-SE analysis of the available CHO genome. The tRNA gene was synthesized using synthetic DNA oligomers according to standard procedures (272). The 5' nucleotide is a cytosine in CHO $tRNA^{Tyr}$, which is a poor substrate for the T7 RNA polymerase, hence a hammerhead ribozyme was ligated between the T7 promoter and the tRNA sequence and cloned into pUC19 vector using BamHI and HindIII restriction sites to yield pUC19-T7 promoter-hammer head ribozyme-CHO $tRNA^{Tyr}$. This plasmid was digested with BstNI to generate 3′ CCA and used as a template for run-off transcription using T7 RNA polymerase. The tRNA transcript was purified on a denaturing 15% polyacrylamide gel and extracted by electrodialysis in 90 mM Tris-borate/2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The tRNA was phenol and chloroform extracted, ethanol precipitated and resuspended in diethylpyrocarbonate (DEPC)-treated ddH_2O.

4.2.5 Aminoacylation assays

All aminoacylation reactions were performed at 37 °C in 144 mM Tris-HCL pH 7.78, 150 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mg/ml BSA, 5 mM ATP, CHO total tRNA, *in vitro* transcribed CHO $tRNA^{Tyr}$ or *E. coli* $tRNA^{Phe}$, L-[U-^{14}C], tyrosine (482 mCi/mmol) or L-[U-^{14}C] phenylalanine (487 mCi/mmol) and aaRSs at concentrations indicated for specific experiments. The reaction was initiated with the

addition of enzyme. Aliquots of reaction mixture were spotted on 3 MM filter paper presoaked in 5% TCA (w/v) at required time intervals, washed in 5% TCA acid, dried and the level of radioactivity was determined by scintillation counting.

4.2.5 Steady-state kinetics

Steady-state kinetic assays were carried out at 25 °C as previously described (30, 299). Reactions were carried out in buffer containing 144 mM Tris-HCL pH 7.78, 150 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 2 mM PP_i. For ATP-PP_i exchange assays to measure amino acid activation (300), concentrations of substrates were varied from 0.5 to 500 μM for Tyr, and 0.5 to 47 mM for Phe. Enzymes were added to a final concentration of 75nM-5μM. Kinetic parameters were calculated by fitting data to the Michaelis-Menten equation using non-linear regression (Kaleidagraph, Synergy Software), and are presented as averages from three independent reactions with the corresponding standard errors.

4.3 Results

4.3.1 Tyr starvation negatively affects cell culture performance

During growth of CHO cell cultures, Tyr starvation was observed in the latter half of fed-batch production with residual concentrations ranging from 0 to 500 μM, while Phe concentrations were maintained between 5 to 9 mM (Figs. 4.1A and B). The routine decrease of Tyr concentration to zero during this period indicated that the intermittent

feed was insufficient to support the culture's utilization of Tyr. A corresponding drop in specific productivity, q_p, on day 9 was observed despite the abundance of Phe in the culture, leading to a 3-fold reduction in q_p by day 16 (Fig. 4.1C). The slope of the titer curve was also lowered as q_p decreased and titer dropped by 1 g/L at day 16 (Fig. 4.1D), and a drop in cell viability followed after day 11 (Fig. 4.1E). In the same experiment, Tyr supplementation was also tested in a separate culture with additions on days 9, 11 and 13. As a result, q_p was maintained at a high level leading to the continual linear rise of the titer curve, while cell viability also significantly improved (Figs. 4.1C-E). Cell growth was not impacted by Tyr starvation as shown by similar integrated viable cell density (IVCD) trends under the two conditions (Fig. 4.1F). Amino acid analysis showed that depletion of Tyr was prevented with the additional Tyr supplementation (Fig. 4.1A). Residual Phe concentrations remained similar in both conditions (Fig. 4.1B). HPLC-MS/MS analyses of the secreted recombinant antibodies produced by these cultures showed multiple Phe misincorporations at a rate of approximately 0.7% per Tyr codon during Tyr starvation (297). In the culture that was not depleted of Tyr, Phe misincorporation at Tyr codons was not observed (<0.01%). Proteomics analysis of related cell lysates revealed 2 fold up regulation of TyrRS in the tyrosine depleted culture after day 7 (297).

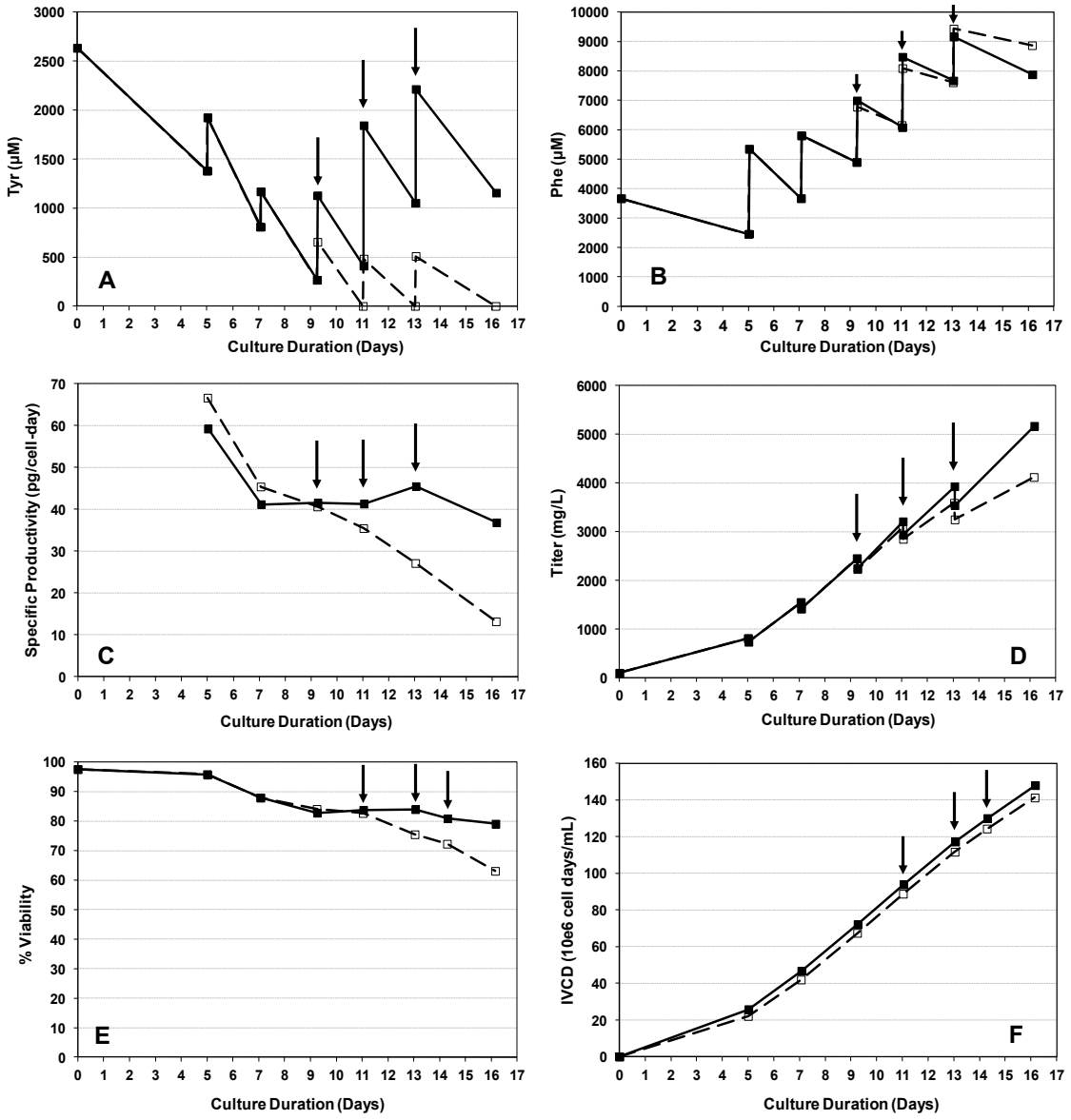


Figure 4.1. Effect of Tyr on CHO culture. (A) Residual Tyr concentration. **(B)** Residual Phe concentration. **(C)** Specific productivity. **(D)** Titer. **(E)** % Viability. **(F)** Integrated viable cell density. Symbols: dashed line (□) – cell culture without Tyr supplementation; solid line (■) – cell culture with Tyr supplementation. Arrows indicate timing of tyrosine addition. This experiment was a collaborative work of Amanda Kano, Mathew Jerums, Paul D. Schnier, Shun Luo, Rohini Deshpande, Pavel V. Bondarenko and Henry Lin.

4.3.2 CHO TyrRS has low specificity for Tyr over Phe

The observed changes in amino acid pools, and the absence of codon context or usage effects on misincorporation rates, suggest that mistranslation may result from errors during the aminoacylation of $tRNA^{Tyr}$ by TyrRS. In order to assess the specificity of amino acid activation by CHO cytoplasmic TyrRS, the corresponding gene sequence (EGW00102) was codon optimized for protein production in *E. coli*. CHO TyrRS was found to misactivate Phe (Fig. 4.2A), and the specificity for Tyr over Phe was found to be 6100:1 (Table 4.1). The amino acid specificity of CHO TyrRS was almost 25-fold lower than that of the well-characterized bacterial TyrRS from *Geobacillus stearothermophilus* (294), indicating a substantial reduction in the discrimination of near cognate amino acid by the eukaryotic enzyme.

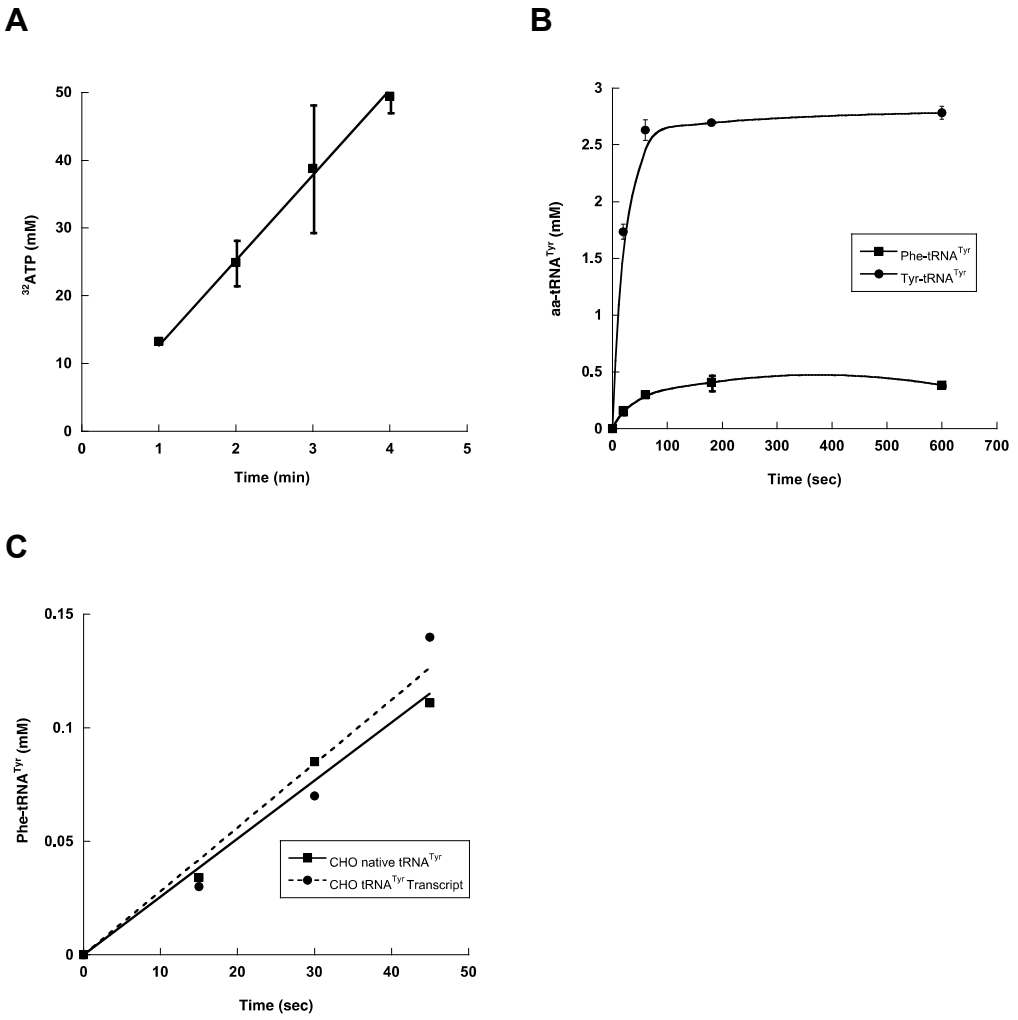


Figure 4.2. Activation and charging of Phe by TyrRS. (A) ATP-PP_i Exchange Assay of CHO TyrRS (250 nM) in the presence of 5 mM Phe at 37 °C. **(B)** Aminoacylation of CHO $tRNA^{Tyr}$ (4 μM) by CHO TyrRS (100 nM) in the presence of 200 μM Phe (■) or 200 μM Tyr (●) at 37 °C. **(C)** Aminoacylation reaction was carried out by CHO TyrRS (100 nM) in the presence of either (6 μM) native $tRNA^{Tyr}$ (■) or (6 μM) *in vitro* transcribed tRNA (●) and 200 μM Phe at 37 °C.

Table 4.2. Steady-state kinetic constants for ATP-[^{32}P]PP_i exchange for CHO cytoplasmic full length (FL) and Mini TyrRS.

	Tyr			Phe[a]	
	K_M (μM)	k_{cat} (s^{-1})	k_{cat} / K_M (s^{-1}/μM)	k_{cat} / K_M (s^{-1}/μM)	Specificity (k_{cat}/K_M)Tyr / (k_{cat}/K_M)Phe
CHO FL TyrRS	15 ± 4	13 ± 2	0.85	1.4×10^{-4}	6200
CHO Mini TyrRS	16 ± 0.6	15 ± 3	0.93	1.2×10^{-4}	7800

[a] k_{cat}/K_M was estimated using sub-saturating Phe concentrations from the slope of the equation, $V = k_{cat}[E][S]/K_M$.

4.3.3 Misacylation of $tRNA^{Tyr}$ by TyrRS

The comparatively low specificity of CHO TyrRS for Tyr over Phe during amino acid activation prompted us to investigate the ability of the enzyme to mischarge $tRNA^{Tyr}$ with Phe. Candidate $tRNA^{Tyr}$ genes encoded in the CHO genome were identified using the tRNAscan-SE software package (301). Two sequences, $tRNA^{Tyr\ 1}$ and $tRNA^{Tyr\ 2}$, were chosen from among the various candidates based on their similarity to known $tRNA^{Tyr}$ genes from related organisms. The two genes were used as templates for *in vitro* transcription, and both resulting $tRNA^{Tyr}$ variants were found to be equally efficient substrates for aminoacylation with tyrosine by TyrRS. CHO TyrRS had a K_M for $tRNA^{Tyr\ 2}$ of 3 ± 1 μM and a k_{cat} of 33 ± 5 s^{-1}, both values within the range typically observed for *in vitro* transcribed tRNAs with aaRSs, and this substrate was used for all further analyses. CHO TyrRS was able to attach Phe to both $tRNA^{Tyr\ 2}$ and CHO total tRNA containing native $tRNA^{Tyr}$ (Fig. 4.2B and C), excluding the possibility that mischarging resulted from the lack of post-transcriptional modifications to the *in vitro* transcribed substrate. Aminoacylation reactions were also performed using *E. coli* $tRNA^{Phe}$ with CHO TyrRS and CHO $tRNA^{Tyr}$ with *E. coli* PheRS to exclude the possibility that the mischarging observed was due to charging of CHO $tRNA^{Tyr}$ by an *E. coli* PheRS contaminant in the CHO TyrRS protein preparation (Figs. 4.3A and B).

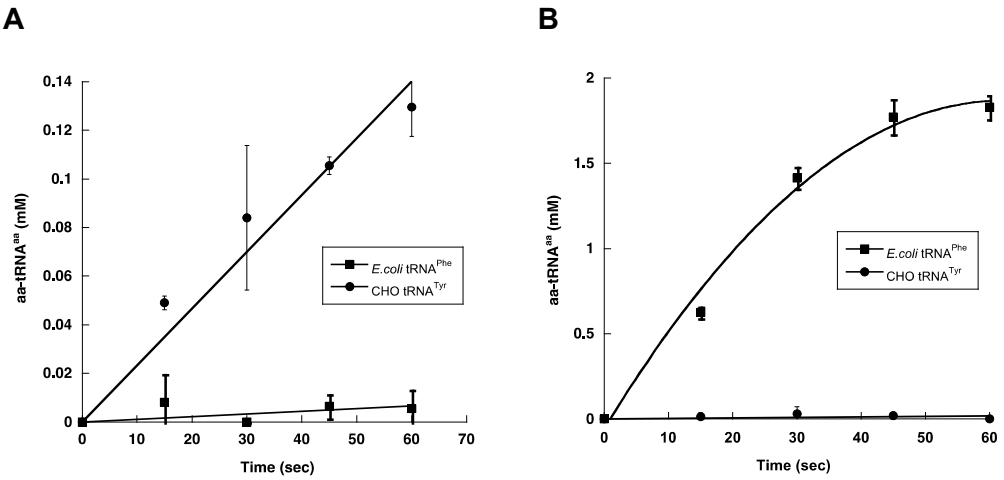


Figure 4.3. Mischarging of Phe by CHO TyrRS is not due to *E. coli* PheRS contamination. Aminoacylation of 6 μM *E.coli* $tRNA^{Phe}$ (■) and 6 μM CHO $tRNA^{Tyr}$ (●) in the presence of 150 μM [^{14}C] Phe, **(A)** CHO TyrRS (100 nM) and **(B)** *E.coli* PheRS (100 nM) at 37 °C.

4.3.4 The endothelial monocyte activating polypeptide II-like domain of CHO TyrRS does not compromise amino acid specificity

During apoptosis mammalian TyrRS is cleaved releasing two fragments, an N-terminal mini-TyrRS and the endothelial monocyte activating polypeptide (EMAP) II-like C-terminal domain, both of which are active cytokines (302). To investigate if mini-TyrRS accumulation impacts mistranslation, the ability of the truncated enzyme to discriminate non-cognate Phe was characterized. Mini-TyrRS misactivated Phe (Fig. 4.4A) and the kinetic parameters for amino acid activation were found to be similar to those for full-length TyrRS (Table 4.2). These results indicate that the presence of the C terminal

EMAP II-domain has no effect on the recognition of cognate Tyr and discrimination against non-cognate Phe, and so would not be expected to affect the level of mistranslation of Tyr codons.

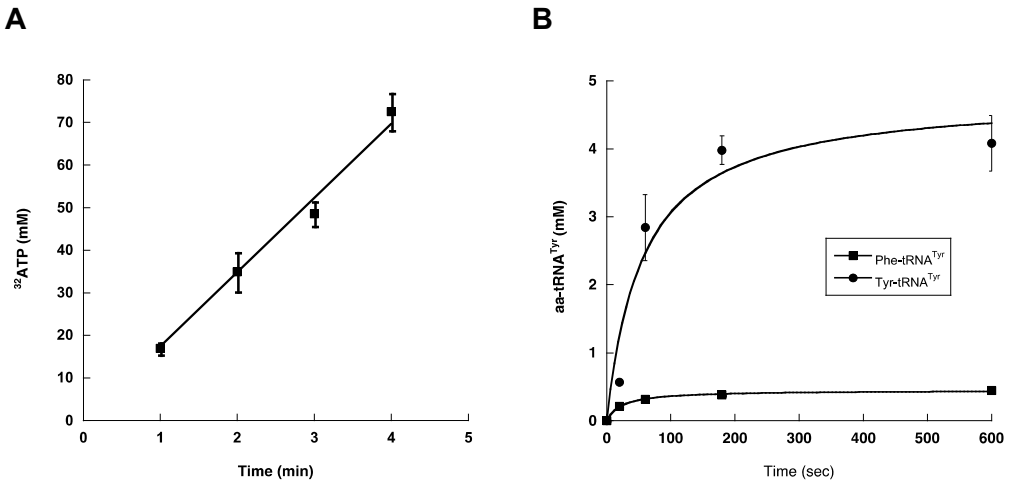


Figure 4.4. Activation and charging of Phe by Mini TyrRS. (A) ATP-PP_i Exchange Assay of CHO Mini TyrRS (250 nM) in the presence of 5 mM Phe at 37°C. **(B)** Aminoacylation of CHO $tRNA^{Tyr}$ (4 μM) by CHO Mini TyrRS (100 nM) in the presence of 200 μM Phe (■) or 200 μM Tyr (●) at 37 °C.

4.3.4 Comparison of bacterial and CHO TyrRS substrate specificity determinants

Comparison of TyrRS active site structures with Tyr bound would provide an ideal means to evaluate how the CHO enzyme evolved to have lower substrate specificity than its bacterial counterpart. Since the crystal structure of the CHO enzyme is not available, we compared the *G. stearothermophilus* (pdb 1tyd) and human (pdb 1q11) TyrRS active sites. The active site of *G. stearothermophilus* TyrRS has been studied in detail; the residues that make hydrogen-bonding interaction with the substrate tyrosine include Tyr 34, Asp176, Tyr169, Asp78 and Gln173. Comparison of the bacterial enzyme to the active sites of human and CHO TyrRS revealed that most of these residues are conserved except for Asp78, the equivalent of which is an Asn residue in eukaryotes (Figs. 4.5A and 4.6). Mutation of CHO Asn82 to Asp had minimal effect on the k_{cat} and K_M of Tyr and Phe, suggesting that the Asn residue is not important for substrate binding in the eukaryotic enzyme (Table 4.3). Most of the hydrophobic interactions in the CHO active site appear conserved except for Ala74 and His77, which are Gly and Thr, respectively, at the corresponding positions in the bacterial enzyme (Fig. 4.5A). Replacement of Ala74 with Gly increased the K_M 7-fold and had no effect on k_{cat} for Tyr. The k_{cat}/K_M for Phe decreased 5 fold. Mutation of His77 to the smaller non-hydrophobic Thr increased the K_M and decreased the k_{cat} by 40-fold (Table 4.3). Substitutions that remove hydrophobic interactions typically lead to a loss of 1-2 kcal/mol of the interaction energy, consistent with the observed increase in the K_M for Tyr. Several other positions not conserved between bacteria and eukaryotes are residues Cys35, His48, Thr 51 and Lys233 in bacterial TyrRS, which interact with ATP during transition state formation

(Figs. 4.5B and 4.6) (303). The CHO TyrRS counterparts of these residues are Trp40, Tyr52, Pro55, and Ser225, all of which lack interactions with ATP (Fig 4.5B and 4.6). Despite the absence of these interactions with ATP in the eukaryotic enzyme, the stabilities of the transition states for Tyr activation have been shown to be virtually identical for the human and *G. stearothermophilus* enzymes. Stabilization of the transition state in eukaryotic TyrRS is potentially achieved by interaction of a potassium ion, which has been shown to replace Lys233, and Pro55 which is believed to replace the Thr51 interaction with ATP (304).

A

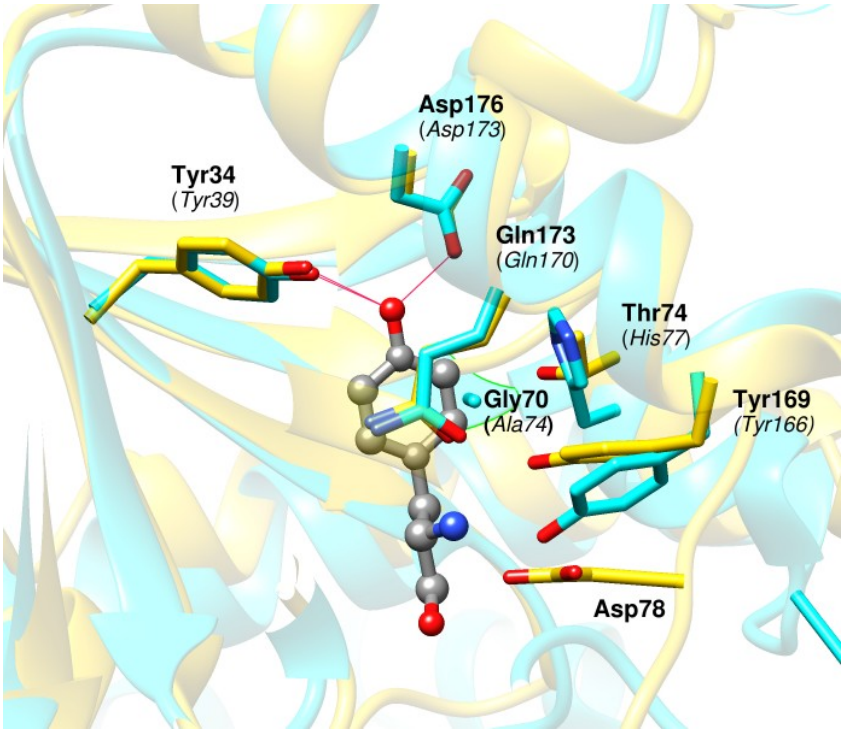


Figure 4.5. Hydrogen bonding and hydrophobic interaction between TyrRS and Tyrosyl-adenylate. (A) The image shows a comparison of the hydrogen bonding interaction between TyrRS and substrate tyrosine. Image corresponds to a superposition of *G. stearothermophilus* and Human TyrRS from pdb files 1tyd (chain E) and 1q11 (chain A). *Gst* TyrRS is in gold , Human TyrRS is in cyan and substrate tyrosine in the active site is in green. Amino acids making hydrogen bonding and hydrophobic interactions are numbered with Human TyrRS numbering in parentheses**. (B)** Hydrogen bonding between the tyrosyl adenylate and TyrRS in the active site of *G. stearothermophilus* with CHO TyrRS numbering in parenthesis. MC, main chain.

Continued

Figure 4.5. Continued

B

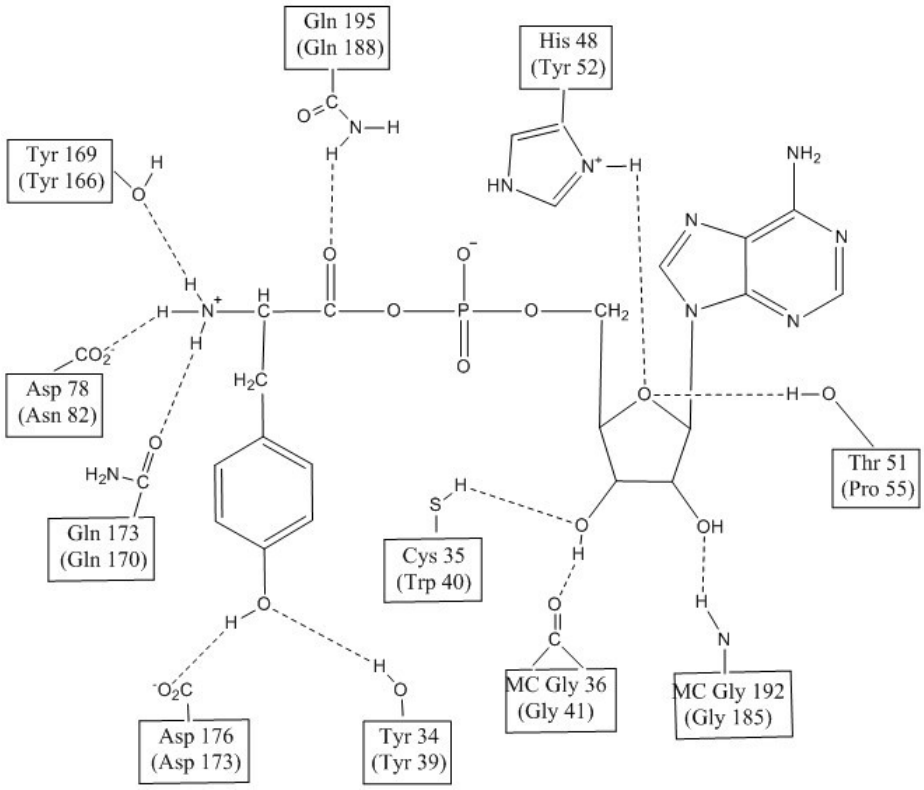
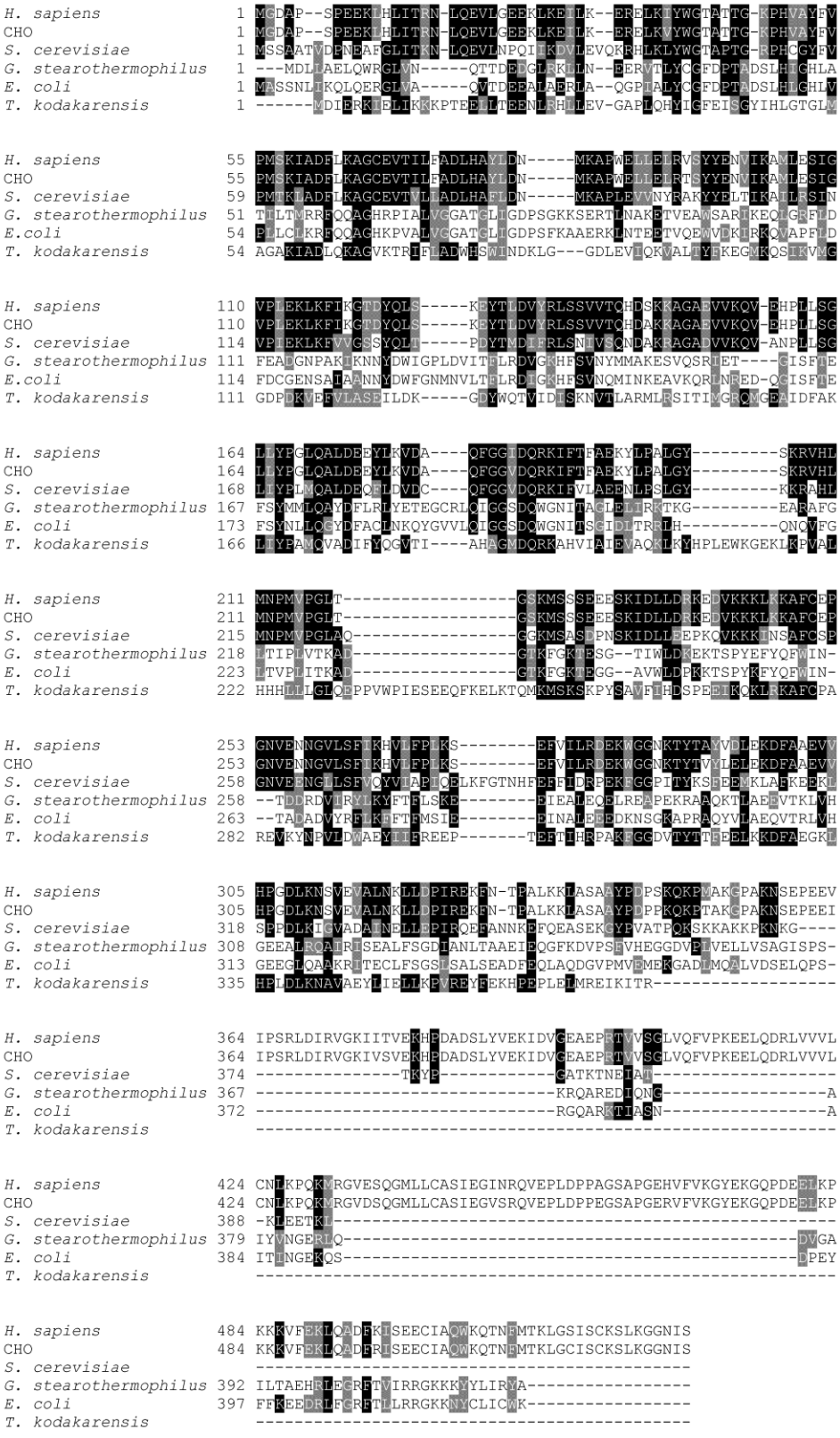


Figure 4.6. Alignment of amino acid sequences of TyrRS. The alignment shown is based on the ClustalW alignment of tyrosyl-tRNA synthetase amino acid sequences from *G. stearothermophilus*, *E. coli, T. kodakarensis, Homo sapiens*, *S. cerevisiae* (cytoplasmic) and CHO. Shaded regions indicate highly conserved amino acids.


H. sapiens
CHO
S. cerevisiae
G. stearothermophilus
E. coli
T. kodakarensis

To determine if differences in ATP binding affect activation of cognate and noncognate substrates, the CHO residues Trp40 and Tyr52 were replaced with their bacterial equivalents, Cys and His, respectively, to generate single amino acid substitutions. The W40C mutation caused a 30-fold decrease of k_{cat} for Tyr activation and a 30-fold increase of K_M. The k_{cat}/K_M for Phe activation decreased 2000 fold, but no significant difference in the specificity for Tyr over Phe was observed indicating that Trp40 is not involved in amino acid discrimination. The Y52H mutation led to a 2-fold increase in K_M and a 20 fold decrease in k_{cat}. The k_{cat}/K_M for Phe decreased 280 fold, thereby increasing the specificity of the enzyme 6 fold. Replacement of Tyr52 may disrupt a possible cation-pi interaction between the aromatic ring of Tyr and the catalytically important potassium ion (Fig 4.7), consistent with the large decrease in k_{cat} observed.

Hydrogen-bonding interactions between bacterial TyrRS residues Asp176 and Tyr34 and the substrate Tyr hydroxyl group help confer amino acid specificity to the enzyme (294, 305). These residues also hydrogen bond to other residues surrounding the inner core of residues that interact directly with substrate; for example, Trp126 and Asn123 are both hydrogen bonded to Asp176 (Fig. 4.8). Trp126 and Asn123 are not conserved across the three domains of life, the equivalent residues in eukaryotes being Tyr and Gly, respectively (Fig. 4.8). The similarity between bacterial and CHO TyrRSs is around 16%, and a number of possible alignments were used, together with available crystal structures, to guide further mutagenesis. Comparing the *G. stearothermophilus* TyrRS and Human TyrRS crystal structure indicated that CHO Gly120 is present at the position corresponding to Asn123, and lacks hydrogen bonding interactions with any

residue which could potentially interact with the OH group of Tyr (Fig. 4.8). Replacing Gly120 with Asn resulted in a 70-fold increase in K_M, a 11-fold decrease in k_{cat} for Tyr, and a 280 fold decreased k_{cat}/K_M for Phe. Replacement of Tyr123 (equivalent to bacterial Trp126) with Trp had minimal effect on the k_{cat} and K_M of Tyr and Phe activation. When additional alignment directed mutations were made (Fig. 4.6) a slight increase in the specificity for the cognate amino acid Tyr was observed. Replacement of Leu125 with the bacterial equivalent Trp had no effect on the k_{cat}/K_M for either Tyr or Phe however changing Asp122 to Asn, led to 5-fold increased k_{cat}/K_M for Phe but had no effect on the K_M and k_{cat} of Tyr activation, leading to 3-fold increases in the specificity of the CHO enzyme. These changes indicate that Asp122 is involved in the discrimination of cognate over non-cognate amino acid.

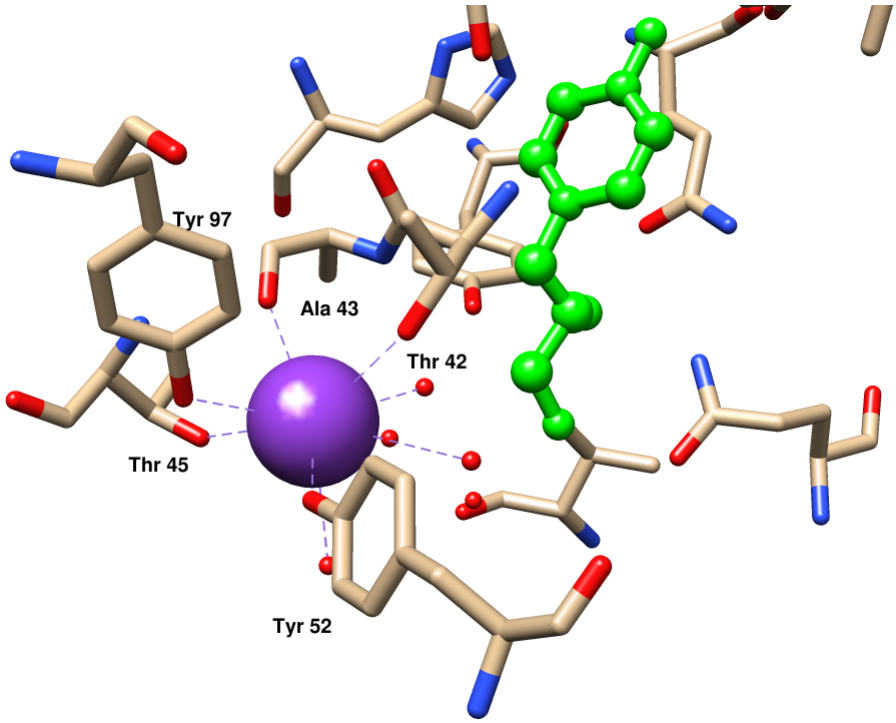


Figure 4.7. Binding site of catalytically important potassium ion in Human TyrRS. The image shows the binding site of potassium ion (purple) with amino acids making possible interactions with the metal ion. The potential metal ligands are labeled, and substrate tyrosine is colored green. Image is made from Human TyrRS crystal structure pdb file 1q11 (chain A).

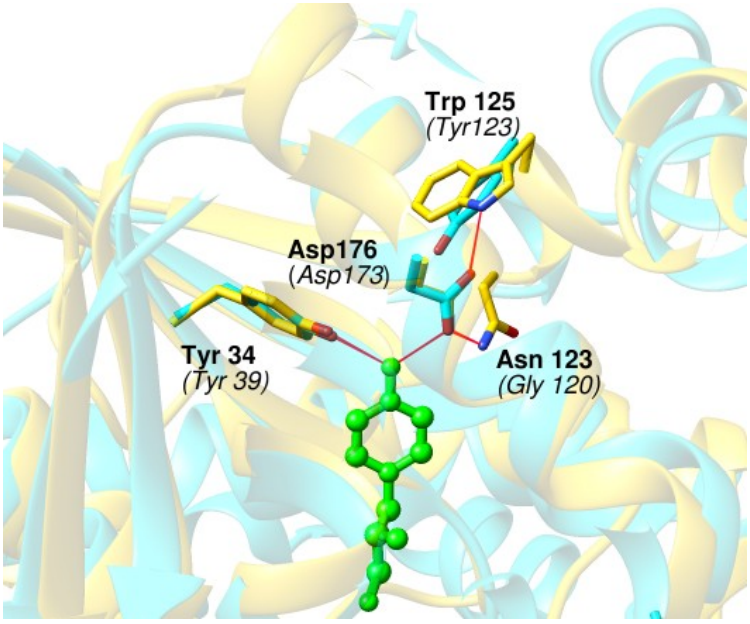


Figure 4.8. Hydrogen bonding network of *G. stearothermophilus* TyrRS Asp176 (CHO Asp173). The image shows the specificity site of TyRS formed by two residues that interact directly with the tyrosine hydroxyl (substrate) by hydrogen bonding: Asp-176 and Tyr-34 with CHO numbering in parentheses. Image corresponds to a superposition of *G. stearothermophilus* and Human TyrRS from pdb files 1tyd (chain E) and 1q11 (chain A). *G. stearothermophilus* TyrRS is in gold, Human TyrRS is in cyan and substrate tyrosine in the active site is in green. Amino acids making hydrogen-bonding interactions are numbered with Human/CHO TyrRS numbering in parentheses

Table 4.3. Steady-state kinetic constants for ATP-[^{32}P]PP_i exchange for CHO cytosolic full length wild type and variant TyrRS.

TyrRS variant	Disrupted contact	Tyr			Phe[a]	Specificity (k_{cat}/K_M)Tyr/ (k_{cat}/K_M)Phe
		K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}/\mu M$)	k_{cat}/K_M ($s^{-1}/\mu M$)	
WT		15 ± 4	13 ± 2	0.85	$1.4 \times 10^{-4} \pm 4 \times 10^{-5}$	6100
W40C		500 ± 200	0.4 ± 0.1	7.9×10^{-4}	$7.3 \times 10^{-8} \pm 9 \times 10^{-9}$	11000
Y52H		36 ± 4	0.70 ± 0.04	0.018	$5 \times 10^{-7} \pm 1 \times 10^{-7}$	36000
A74G	Hydrophobic	100 ± 45	12 ± 6	0.11	$3 \times 10^{-5} \pm 1 \times 10^{-5}$	4100
H77T	Hydrophobic	640 ± 50	0.30 ± 0.02	4.7×10^{-4}	$7.3 \times 10^{-8} \pm 1 \times 10^{-9}$	6500
N82D	H bonding with substrate	40 ± 15	5 ± 2	0.12	$1.9 \times 10^{-5} \pm 2 \times 10^{-6}$	6300
G120N	2° H bonding	1100 ± 50	1.2 ± 0.2	1.2×10^{-3}	$5.0 \times 10^{-7} \pm 4 \times 10^{-8}$	2400
Y123W	2° H bonding	40 ± 2	7.3 ± 0.8	0.18	$2.3 \times 10^{-5} \pm 6 \times 10^{-6}$	7800
D122N	2° H bonding	15 ± 2	9 ± 0.7	0.6	$3 \times 10^{-5} \pm 1 \times 10^{-5}$	20000
L125W	2° H bonding	12 ± 0.4	8 ± 1	0.72	$1.3 \times 10^{-4} \pm 1 \times 10^{-5}$	6000

[a] k_{cat}/K_M was estimated using sub-saturating Phe concentrations from the slope of the equation, $V = k_{cat}[E][S]/K_M$.

4.4 Discussion

4.4.1 Amino acid imbalance increases translation error rates

AaRSs maintain translational fidelity either by accurate binding of the correct amino acid or proofreading and editing of non-cognate products. Previous studies have shown that errors in translation can occur randomly at a rate of about $4x10^{-4}$ to $5 x10^{-5}$ per codon or 0.005 –0.04% per site under normal growth conditions (306, 307). Various studies have suggested that error rates in protein synthesis can increase under a variety of conditions such as oxidative stress, (52, 296, 308), changes in codon bias (309, 310), genetic heterogeneity (311), heterologous overproduction (312) and amino acid starvation (307). To determine the rate and extent of mistranslation under different conditions accurate detection and quantification of amino acid misincorporation is critical (313, 314). Aside from the technical challenges, measuring mistranslation is further complicated by the expectation that misincorporation could lead to protein misfolding and subsequent degradation by the cellular protein quality control machinery. This potential problem was addressed by measuring misincorporation rates in a secreted antibody, which revealed Phe substitutions at 25 out of 30 Tyr codons in the recombinant protein at an overall error rate of approximately 0.7% per residue during Tyr starvation (Fig. 4.1A; (297)). The higher than expected level of Tyr to Phe misincorporation was effectively suppressed by supplementing the media with Tyr, indicating that the increase in error rate was due to an imbalance in cellular amino acid pools. Amino acid imbalances are encountered by mammalian cells under various conditions such as, for example, bacterial

infections that trigger acute intracellular amino acid starvation due to host membrane damage (315) or cancer tumor microenvironments that become nutritionally deprived due to rapid cell proliferation. Our findings suggest that under such growth conditions protein synthesis error rates may also rise significantly, but whether this would be expected to have beneficial or detrimental effects on the cell is unclear and may depend on the level of mistranslation in a particular system (314).

4.4.2 Misincorporation of Phe at Tyr codons is due to mischarging of $tRNA^{Tyr}$

It has previously been proposed that aaRSs should exhibit a selectivity of at least 3000 fold for cognate over non-cognate amino acids in order to maintain error rates below 10^{-4} during translation (316). Selectivity is the product of the specificity of the aaRS and the ratio of cognate vs non-cognate amino acid (22). The specificity of bacterial TyrRS for tyrosine over Phe is 10^{-5} (294) and the Phe:Tyr ratio in a dividing bacterial cell is typically around 1.9:1 (257). These two factors combined give a rate of misincorporation comparable to other amino acid pairs and show that, in the absence of an editing mechanism, bacterial TyrRS can discriminate between Tyr and Phe by simple preferential binding and activation. In the case of CHO TyrRS, steady-state kinetic analysis revealed a lower specificity of 6100 for Tyr over Phe compared to the value of 150,000 reported for bacterial TyrRS. Under Tyr starvation the relative concentration of Phe to Tyr was as high as 18:1 thereby decreasing the selectivity for cognate over non-cognate amino acid to around 340:1. Hence, while the selectivity of CHO TyrRS is above the threshold of 3000:1 under normal growth conditions, it is significantly lower during Tyr limitation

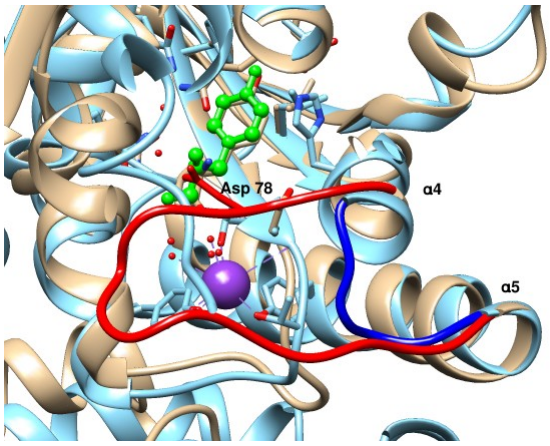
and results in mistranslation. This increased susceptibility to translation errors observed in higher eukaryotes compared to bacteria suggests that protein synthesis quality control has evolved with different constraints in each kingdom. On a practical level our data also illustrate the utility of determining optimal cognate to non-cognate amino acid concentrations in the medium in order to overcome poor aaRS specificity and mitigate tRNA mischarging. This approach of understanding specificity requirement may be applicable to reduce other amino acid misincorporations caused by tRNA mischarging such as Ser→Asn in CHO produced antibodies (307).

4.4.3 Divergence in amino acid discrimination between bacterial and eukaryotic TyrRS

Bacterial TyrRS displays high specificity towards its cognate amino acid Tyr as compared to the noncognate substrate Phe (294). Our results demonstrate that higher eukaryotic TyrRSs have evolved to have a lower specificity for Tyr over Phe than their bacterial counterparts (Table 4.2). Although the active site residues in *G. stearothermophilus* and human TyrRS are largely conserved, several key differences exist between the two enzymes. One important H-bonding interaction missing from the eukaryotic enzyme is mediated in the bacterial system by Asp78, which is located in a loop region between helix α4 and helix α5 (Fig 4.9). This loop is located at the entrance to the tyrosine-binding site and undergoes a substantial conformational change upon binding of the substrate. This loop region of the bacterial enzyme is also more hydrophilic than its eukaryotic counterpart (Fig 4.9). In the eukaryotic enzyme, the loop

provides a hydrophobic lid over the tyrosine-binding pocket and the conformational change is thought to play a role in sequestering the activated amino acid from water during the catalytic reaction (317). The active site of the eukaryotic TyrRS also has two hydrophobic interactions, via His77 and Ala74, which are missing in the bacterial enzyme. His77 was found to be important for substrate binding (Table 4.3), illustrating how the eukaryotic enzyme has evolved in a way that may improve binding of the more hydrophobic Phe in the active site of CHO TyrRS. In addition to differences in direct enzyme-substrate interactions, changes in the hydrogen bonding network are also evident in the eukaryotic TyrRS. Secondary interaction between the catalytically important Asp173 and residue Asp122 in the eukaryotic enzyme was found to contribute to discrimination between the cognate vs the noncognate substrate. This residue is located on helix η6 and the loop connecting helix η2 and α6 and could influence specificity by, for example, inducing a conformational change in the loop itself or by altering the protein backbone conformation as shown recently for aspartate aminotransferase (318). Mutation of Tyr52, which is located in the ATP binding region, removes a potential interaction with the catalytically important potassium ion which must be replaced by an interaction somewhere else in the protein. This mutation decreases Phe activation more than Tyr, thereby generating a TyrRS variant with 6 fold higher specificity for the cognate amino acid. The future deployment of aaRS variants with improved amino acid specificity, as described here, may help to significantly reduce elevated amino acid misincorporation during heterologous protein over production.

Aminoacyl-tRNA synthetases have drawn interest as potential targets for the development of new antibiotics. For example, a series of related competitive inhibitors have been identified that bind 40,000-fold more tightly to *Staphyloccocus aureus* than to *Saccharomyces cerevisiae* TyrRS (319, 320). The selectivity of these inhibitors is consistent with the differences described here between the active sites of the bacterial and eukaryotic enzymes, and supports the utility of TyrRS as a target for antimicrobial therapeutics.



Peptide	Charge	Attribute
IGDPSGKKSERTLNA	1	Basic

Peptide	Charge	Attribute
DNMKAP	0	Neutral

Figure 4.9. Comparison of *G. stearothermophilus* and Human TyrRS active sites. Superimposition of the active sites of *G. stearothermophilus* and Human TyrRS (left). Bacterial loop I78-A90 colored in red and the eukaryotic counterpart colored in blue. Hydrophobicity analysis of the loop between helices α4 and α5 of *G. staerothermophilus* TyrRS and Human TyrRS (right). Red color represents acidic residues (D and E), Blue: basic residues (R,K and H) Green: hydrophobic uncharged residues (F,I,L,M,V,W,A and P) black other residues (G,S,T,C and Q).

4.4.4. Evolution of quality control mechanisms in higher eukaryotes

Accumulation of mischarged tRNAs, and their use in translation, is normally minimized by cellular quality control process such as editing (22, 41). Editing does not target all mischarged tRNAs; for example while Ala-tRNA^{Pro} and Tyr-tRNA^{Phe} are subject to quality control (41, 321), Ser-tRNA^{Arg} and Phe-tRNA^{Tyr} are not, as the respective aaRSs lack any known editing activities. The observed increases in amino acid misincorporation are not uniform under amino acid starvation conditions, suggesting that mistranslation of some amino acids is more detrimental to the cells than others, for which more stringent quality control mechanisms have evolved. In the specific example studied here, the broader role of tyrosine in eukaryotes compared to bacteria may also exert an important effect on the evolution of quality control. It has been shown previously that during evolution of higher eukaryotes a significant selection pressure existed for loss of Tyr, notably in protein subsets that are not known to be substrates for tyrosine phosphorylation (322). The loss of Tyr in higher eukaryotic protein evolution might have also have been facilitated by a reduction in the accuracy of tyrosine decoding, which could most readily be achieved in a codon-specific manner by reduced quality control at the level of aminoacylation as observed here for CHO TyrRS.

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Chapter 5:

Outlook

AaRSs have evolved to use additional domains acquired during evolution. These domains are required for RNA recognition, proofreading activities and protein–protein interactions which have numerous and diverse roles, thereby expanding aaRS functions in and beyond translation of mRNA. Formation of aaRS complexes mediated by these unique extensions is one of the aaRS characteristics observed in archaea and eukaryotes. While the mammalian multi-synthetase complex (MSC) has been extensively studied, its function in translation remains unclear. The observation of enhanced aa-tRNA synthesis by aaRS within MSCs [Chapter 2, (323)] provides a possible mechanism to increase the rate of cellular protein synthesis. Complex formation between an elongation factor and an aaRS has been hypothesized to play a role in direct channeling of aminoacyl-tRNAs from aaRS to the site of protein synthesis and shuttling of the uncharged tRNAs exiting the ribosome back to the complex. The presence of 30S ribosomal proteins, IF2, IF2B, EF2, in the LeuRS interactome, and the detection of several aaRS activities enriched in polysomes (chapter 2), support the channeling model and indicate the possibility of

association of the MSC with the translation machinery. Direct association of the MSC with the ribosomal proteins could increase translational efficiency by preventing tRNA diffusion away from the ribosome by binding the deacylated tRNA, recharging and channeling the charged tRNA to elongation factors in proximity of the ribosome. Further experimentation is needed to show the physical association of the MSC with the ribosome. Also, it would be of great interest to investigate the physiological role of the MSC in translation by studying the cellular effects of disrupting proper MSC assembly by targeted disruption of protein-protein interactions between specific aaRSs.

AaRS fidelity is achieved through discrimination at the aminoacylation site as well as through additional editing domains in some aaRSs. However, it is becoming increasingly clear that these quality control mechanisms have evolved differently in different organisms and that the requirement for quality control also depends on various environmental conditions. For example, several *Mycoplasma* species lack editing mechanisms associated with some of their aaRSs like LeuRS, PheRS and/or ThrRS (60). The AT-rich genome of this organism was proposed to act as a non-specific factor facilitating a shift in the amino acid composition favoring amino acids containing AT rich codons and also effecting aaRSs by favoring substitutions at various key synthetic and editing site residues. The evolutionary advantage of these error-prone proteomes is unclear, but it has been proposed that, reduction in discrimination between similar amino acids may provide a potential mechanism for these organisms to adapt to the amino acid pools in the organism. Such flexibility would enable the organism to maintain viable translation without completely losing structural and functional integrity of proteins. Also,

because many of these organisms are obligate intracellular pathogens, misincorporation of similar amino acids has been proposed to increase antigen diversity, thereby allowing the organism to evade host defense system.

The diversity of these discrimination mechanisms has also been observed in the activation and editing of the similar amino acids Phe and Tyr. While yeast cytoplasmic PheRS displays low specificity and relies on an editing mechanism to clear mischarged $tRNA^{Phe}$, mitochondrial PheRS has evolved to have high Phe specificity. In comparison, *E. coli* PheRS displays both specificity and editing activity against mischarged Tyr-$tRNA^{Phe}$ (58). Interestingly, these editing mechanisms are found to be dispensable to the cell under normal growth conditions and hence raise the question about the selective pressures that dominate the evolution of different quality control mechanisms in different organisms. The observation that in eukaryotes, cytoplasmic PheRS post-transfer editing is needed to protect the proteome from *p*-Tyr misincorporation in contrast to *E. coli* PheRSs that use post-transfer editing to prevent *m*-Tyr and *p*-Tyr misincorporation (Chapter 3), highlights the possibility that many dispensable editing mechanisms might have evolved for alternate substrates. A further study of the total proteome is required to identify other non-proteinogenic amino acids that might act as substrates in protein synthesis. A number of non-proteinogenic amino acids for example homocysteine, norleucine, α-aminobutyrate which are precursors in various biosynthetic processes and other amino acids like L-DOPA, and leucine hydroxide generated as a result of oxidative stress have been shown to be used as substrates in protein synthesis (258, 289, 290). These

observations indicate a link between quality control mechanisms, metabolism and stress response in the cell.

While PheRSs have evolved to exhibit various levels of quality control, TyrRSs exhibit no pre or post–transfer editing activity and rely solely on high substrate specificity for Tyr as a quality control mechanism. The observation of 25 fold lower specificity in higher eukaryotic TyrRS as compared to bacterial TyrRS further supports the notion that protein synthesis quality control has evolved with different constraints in each kingdom (Chapter 4). This low specificity becomes a problem under nutrient starvation conditions, which lead to the accumulation of non-cognate Phe at Tyr codons in the proteome of eukaryotic cells. The divergent quality control of activation of similar amino acids Phe and Tyr, which differ only by a single hydroxyl group, suggests that Tyr misincorporation is potentially more detrimental to the cell compared to Phe as observed by the stringent quality control mechanisms displayed by PheRS. This hypothesis could be explained by the unique selection pressure for PheRS and TyrRS quality control in bacteria and eukaryotes. The broader role of tyrosine in eukaryotes compared to bacteria may also exert an important affect on the evolution of quality control. It has been shown that during evolution of higher eukaryotes with the expansion of the tyrosine kinase family of proteins, there has been a correlated reduction in the number of Tyr residues encoded by the genome (322). In these higher eukaryotic cells, phosphorylation of Tyr is used as regulator of various intracellular processes like cell cycle control (324), gene regulation and transcription (325), Angiogenesis (326), cell adhesion, spreading, migration and shape (327). Changes in tyrosine kinase activity are implicated in

numerous human diseases, including cancers, diabetes, and pathogen infectivity (328). Also, many tyrosine kinases are implicated in human cancer either through gain of function mutations or overexpression of these kinases (329, 330). Hence, loss of Tyr sites in the proteome would lead to optimization of phosphotyrosine signaling networks and a strong selective pressure against the miscoding of Tyr for Phe during protein synthesis, and thus a selective pressure to maintain a high level of quality control in the corresponding PheRS. Alternatively, it could be possible that substitution of Tyr with Phe would help regulate a variety of these processes in response to nutrient stress. Further studies are required to understand whether the low amino acid specificity gives some sort of selective advantage to cell. Also, studies on tyrosine phosphorylation and the effect of these substitutions on the signaling pathways would help prove or refute this hypothesis.

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