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Non-canonical roles of tRNAs and tRNA mimics in bacterial cell biology

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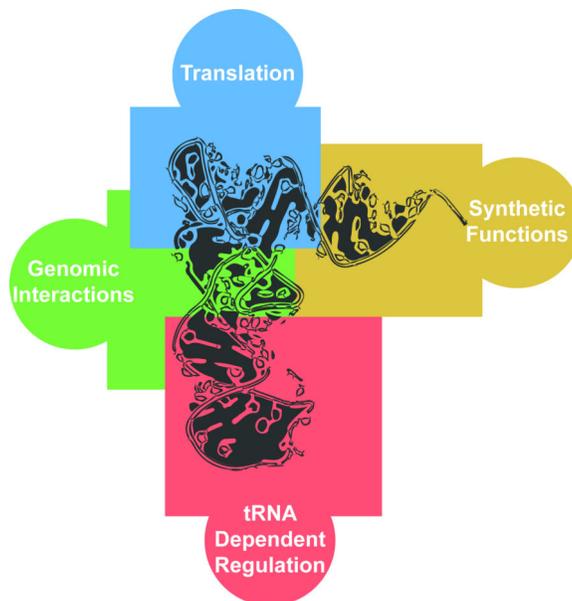
Abstract

Transfer RNAs (tRNAs) are the macromolecules that transfer activated amino acids from aminoacyl-tRNA synthetases to the ribosome, where they are used for the mRNA guided synthesis of proteins. Transfer RNAs are ancient molecules, perhaps even predating the existence of the translation machinery. Albeit old, these molecules are tremendously conserved, a characteristic that is well illustrated by the fact that some bacterial tRNAs are efficient and specific substrates of eukaryotic aminoacyl-tRNA synthetases and ribosomes. Considering their ancient origin and high structural conservation, it is not surprising that tRNAs have been hijacked during evolution for functions outside of translation. These roles beyond translation include synthetic, regulatory and information functions within the cell. Here we provide an overview of the non-canonical roles of tRNAs and their mimics in bacteria, and discuss some of the common themes that arise when comparing these different functions.

Graphical Abstract

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Introduction

All currently known genetic information is coded in nucleic acids (RNA or DNA). In order to be functional, much of this genetic information must first be decoded into proteins that are composed of amino acids that do not have any specific affinity to the message coded in nucleic acids (Crick, 1958; Crick, 1970; Francklyn and Minajigi, 2010). In order to translate this genetic information cells need adaptors, molecules that can translate nucleic acid into amino acid sequences (Crick, 1958; Hoagland et al., 1958). This adaptor function is performed by aminoacyl-transfer RNAs (aa-tRNA), which have a nucleic acid portion composed of a highly structured transfer RNA (tRNA) of about 80 nucleotides. The second part of an aa-tRNA molecule is composed of a single amino acid bound through an ester bond to the 2' or 3' OH of the 3'-end of the tRNA. The secondary structure of the tRNA is composed of an acceptor arm that carries the amino acid, the anticodon arm where there is an anticodon triplet which recognizes codons on mRNA during translation, and the deoxyuridine, TΨC and variable arms (D, T, and V arms respectively) (Fig. 1) (Ladner et al., 1975; Giegé and Frugier, 2000; Marck and Grosjean, 2002). Transfer RNAs are functionally grouped into families of isoacceptors, which may differ in sequence, but all of which carry a unique amino acid for protein synthesis. This amino acid is added by aminoacyl-tRNA synthetases (aaRS), a group of enzymes that specifically recognize and activate a particular amino acid and transfer it to the correct tRNA. This reaction is central to the process of translation as it ensures that the correct amino acid is transferred to the nascent peptide (Cavarelli and Moras, 1993; Schimmel et al., 1993; Ibba et al., 2005). In order to select the correct tRNAs, aaRS recognize specific nucleotides called “identity elements”. These nucleotides are scattered throughout the tRNA structure, but are usually concentrated in the anticodon loop and the acceptor stem (Beuning and Musier-Forsyth, 1999). Ribosomes also contact tRNAs throughout their surface and require them to be flexible molecules. Nevertheless, with the exception of discrimination between initiator and elongator tRNAs,

interactions with anticodon nucleotides have a predominant role in selection of the correct tRNA. Additionally, other regions of the tRNA have a role in compensating for the different affinity of the diverse anticodons and amino acids that are used in translation either directly interacting with the ribosome or allowing for optimal flexibility (Dale and Uhlenbeck, 2005; Khade and Joseph, 2010; Shepotinovskaya and Uhlenbeck, 2013).

Phylogenetic studies and the observation that tRNA structure and the genetic code are essentially maintained in all known organisms, suggest that tRNAs (or their direct and structurally similar predecessor) originated at the time of the last universal common ancestor or earlier (Schimmel et al., 1993; Di Giulio, 1995; Di Giulio, 2006; Sun and Caetano-Anollés, 2008; Moura et al., 2010). Precursors of tRNA might even predate the existence of proteins and could have had a catalytic or metabolic role outside of translation in the “RNA world” (Rodin et al., 2011; de Vladar, 2012; Morgens, 2013). Regardless of their exact origin, the high degree of similarity between extant tRNAs probably derives from their role in a very complex machinery where changes in one component could necessitate modification of other parts of the genetic code and/or translation apparatus to maintain function. An example of this can be observed in the mitochondria of several metazoans where many tRNAs have lost either their T or D arms. In the cases that have been studied in more detail, it was observed that EF-Tu, rRNA, ribosomal proteins and aaRS coevolved to allow the recognition of these non-canonical tRNAs (Watanabe et al., 2014). Changes in the “sense” of codons (where they are reassigned to encode a different amino acid) also require major modifications as the appearance of this new sense for a codon would otherwise force the introduction of amino acids in an erroneous context. For instance, comparative genomic studies have suggested that Leu CUG codon reassignment to Ser in several *Candida* and *Debaryomyces* fungi led to a drastic reduction in the usage of these codons (Moura et al., 2010). Replicating this codon reassignment in the model organism *Saccharomyces cerevisiae* resulted in decreased fitness in several growth media and decreased thermal stability for at least one protein. Nevertheless, these experiments also showed a great growth advantage in some culture conditions (e.g. a 500% growth advantage in the presence of copper sulfate). This change in niche preference and a probable low level of misincorporation in the original conditions may have allowed the fixation of these changes to the genetic code (Mateus et al., 2013). Other codon reassignments may have required the prior elimination of the original codon from the genetic code, for instance due to abnormally high or low CG usage in the genome (Moura et al., 2010). Although such tRNA-dependent modifications to the translation apparatus are rare, they probably had an important role in the original establishment and/or expansion of the genetic code by facilitating the coding of new amino acids (Moura et al., 2010; Rodin et al., 2011; de Vladar, 2012). Beyond its integral role in defining and translating the genetic code, tRNA has also evolved a surprisingly wide range of alternative functions (Fig. 2). The aim of this review is to analyze the pathways that have evolved to utilize tRNAs, sometimes by hijacking them to function beyond the translation machinery. Emphasis will be placed on bacterial systems, and examples from other organisms will be mentioned when relevant.

aa-tRNA based synthesis beyond translation

Modification of macromolecules

Many different aa-tRNAs are used outside of translation to provide activated amino acids for a wide variety of purposes. These include the synthesis of several metabolites as well as the modification of macromolecules (RajBhandary and Söll, 2008; Banerjee et al., 2010; Francklyn and Minajigi, 2010; Dare and Ibba, 2012; Katz and Orellana, 2012a; Belin et al., 2012; Raina and Ibba, 2014). Some of the earliest described examples of such roles outside of translation are related to modification of cellular envelopes (RajBhandary and Söll, 2008; Francklyn and Minajigi, 2010). For example, the addition of amino acids to phosphatidylglycerol through ester bonds to the glycerol moiety and synthesis of certain peptide cross bridges for peptidoglycan both require aa-tRNA substrates (RajBhandary and Söll, 2008). Modification of lipids is used to change the membrane charge distribution, thereby protecting the cell against some antibiotics or bactericidal peptides produced by competing species (Dare and Ibba, 2012; Raina and Ibba, 2014; Goldfine, 2014). Lipid aminoacylation also participates in resistance to osmotic or pH stresses (Dare and Ibba, 2012; Goldfine, 2014) and might even have a role in bacterial replication by modulating the ability of DnaA to exchange ADP with ATP (Ichihashi et al., 2003). In addition to lipid modification, aa-tRNAs can also be used as substrates for the addition of amino acids to peptides in bacteria. This includes the synthesis of the pentapeptide bridge from peptidoglycan peptide by MurM or Fem proteins, modification of the amino terminus in proteins starting with basic amino acids by leucyl/phenylalanyl transferases (L/F-transferases; in eukaryotes Arg-tRNA-protein transferase modifies proteins with Arg) and the synthesis or modification of some small peptides using enzymes like AlbC, PacB or VlmA (Berger-Bächi and Tschierske, 1998; Garg et al., 2008; Zhang et al., 2011; Dougan et al., 2012; Dare and Ibba, 2012; Belin et al., 2012; Shepherd and Ibba, 2013b; Raina and Ibba, 2014). These amino acid additions to peptides have diverse functions. The peptidoglycan bridge changes the structural properties of the cell wall, enhancing antibiotic resistance and binding of some extracellular proteins (Berger-Bächi and Tschierske, 1998; Guan et al., 2004; Scheffers and Pinho, 2005; Dare and Ibba, 2012; Shepherd and Ibba, 2013b). By contrast, modification of the amino terminus of cytoplasmic proteins modulates the half-life of specific proteins by targeting them for recognition by ClpS and subsequent degradation by ClpAP protease in bacteria or by UBR proteins and subsequent degradation by the 26S proteasome in eukaryotes (Dougan et al., 2012; Raina and Ibba, 2014). Finally, small peptides synthesized using the activated amino acid on aa-tRNA as a precursor correspond to secondary metabolites with diverse physiological functions ranging from antibiotics and inter-cellular signaling molecules to metal chelators (Garg et al., 2008; Zhang et al., 2011; Belin et al., 2012).

In all cases that have so far been studied, the specificity for aa-tRNA in the modification of macromolecules depends primarily on recognition of the amino acid with a minor but relevant role for the tRNA, particularly the acceptor stem. For instance, FemX from *Weissella viridescens* uses Ala-tRNA^{Ala} as substrate, excluding from its active site any amino acid that is bigger than Ala. The only aa-tRNA carrying a smaller amino acid, Gly-tRNA^{Gly}, is excluded by recognition of tRNA “anti-determinants”, that is, the exclusion of

any tRNA that presents a specific sequence (in this case base pair C2-G71) that is absent from the natural substrate of the enzyme (Fonvielle et al., 2009). Base pairs that are outside of the acceptor stem of tRNA, or even 3 to 4 base pairs away from the amino acid, have a negligible effect on the aa-tRNA specificity of FemX (Villet et al., 2007; Fonvielle et al., 2009; Fonvielle et al., 2013). Studies on aminoacyl-phosphatidylglycerol synthases (MprF or aa-PGS) that catalyze lipid modification (Gould et al., 1968; Roy and Ibba, 2008; Hebecker et al., 2011; Dare and Ibba, 2012), L/F-transferases that modify the amino terminus of proteins tagging them for degradation (Suto et al., 2006; Watanabe et al., 2007; Wagner et al., 2011; Fung et al., 2014), and AlbC that catalyzes the synthesis of a cyclic dipeptide (Bonfond et al., 2011; Sauguet et al., 2011; Moutiez et al., 2014) all showed similar dependence on acceptor stem sequences. From this perspective, AlbC is particularly interesting as the enzyme uses two aa-tRNAs that bind sequentially to different regions of the enzyme. The enzyme is more specific for the recognition of the first aa-tRNA (where it uses only one amino acid) than for the second aa-tRNA (where it uses one amino acid preferentially, but accepts others). In the case of AlbC, recognition of the second aa-tRNA depends strongly on the identity of the first base pair of the tRNA acceptor stem (G1-C72 in AlbC substrates), while the identity of these nucleotides is not relevant for the recognition of the first aa-tRNA substrate (Moutiez et al., 2014).

Synthesis of small metabolites

In addition to their roles in the synthesis or modification of macromolecules, tRNAs participate in the production of several much smaller metabolites. Beside the synthesis of dipeptides mentioned above, tRNAs have central roles in the synthesis of tetrapyrroles (such as heme or chlorophyll) and several amino acids. Many organisms lack the complete set of aaRSs required to aminoacylate every tRNA isoacceptor with the corresponding amino acid. In these organisms some tRNAs are aminoacylated in an indirect pathway where the cognate tRNA is first “mis-acylated” with a precursor of the correct amino acid that is subsequently modified while on the tRNA. These indirect pathways have been observed for the synthesis of glutamine on tRNA^{Gln} (in all known archaea, as well as in most bacteria and eukaryal organelles), asparagine on tRNA^{Asn} (in several bacteria and archaea), formylmethionine on tRNA^{fMet} (in bacteria and eukaryotic organelles), cysteine on tRNA^{Cys} (in some archaea) and selenocysteine on tRNA^{Sec} (in some bacteria, archaea and eukarya) (Ibba et al., 2000; O’Donoghue et al., 2005; Sheppard et al., 2008; Yuan et al., 2008; Katz and Orellana, 2012a). In several organisms the main function of these pathways is to provide aa-tRNA for the synthesis of proteins. Furthermore, some organisms lack alternative pathways to synthesize these amino acids and for them, tRNA dependent synthesis is the sole means to produce these metabolites (Sauerwald et al., 2005; O’Donoghue et al., 2005; Katz and Orellana, 2012a; Mladenova et al., 2014). The indirect aminoacylations of “amide” (Gln and Asn) tRNAs were among the first of these pathways to be described. In these examples, tRNA^{Gln} or tRNA^{Asn} are first aminoacylated with the “acid” amino acid (Glu or Asp) by a non-discriminating aaRS (ND-GluRS or ND-AspRS). In some organisms, these non-discriminating enzymes aminoacylate the tRNAs for both acid (tRNA^{Glu} or tRNA^{Asp}) and amide (tRNA^{Gln} or tRNA^{Asn}) amino acids, but in other organisms there are specialized enzymes for each kind of tRNA. In a second step, a tRNA dependent amidotransferase (AdT) catalyzes the formation of the amide (Gln or Asn) on the tRNA (Katz and Orellana,

2012a). The other indirect pathways follow similar steps, first adding a precursor (phosphoserine (Sep) on tRNA^{Cys}, Met on tRNA^{fMet} or Ser on tRNA^{Sec}) that is converted into the final product in one or two additional reactions (catalyzed by Sep-tRNA:Cys-tRNA synthase (SepCysS) for Cys-tRNA^{Cys}, methionyl-tRNA formyltransferase (FMT) for fMet-tRNA^{fMet} and selenocysteine synthase (SelA) in bacteria or phosphoseryl-tRNA kinase (PSTK) plus Sep-tRNA:Sec-tRNA synthase (SepSecS) in archaea and eukarya for Sec-tRNA^{Sec}). In all these pathways, the production of a “mis-acylated” tRNA as an intermediary could potentially allow the mis-incorporation of amino acids during translation. This is prevented mainly by two processes: 1) the ability of translation elongation factors and, possibly the ribosomal A site, to partially discriminate tRNAs, binding preferentially the correctly acylated ones (LaRiviere et al., 2001; Dale and Uhlenbeck, 2005; Yuan et al., 2008; Katz and Orellana, 2012a) and 2) the formation of complexes between the non-discriminatory aaRS and the modifying enzyme from the second step of the pathway that sequester the mis-acylated tRNAs (Bailly et al., 2007; Zhang et al., 2008; Rampias et al., 2010; Katz and Orellana, 2012a). Additionally, in some cases channeling is not required as kinetic competition between the amino acid modifying enzyme and elongation factor prevents accumulation of mis-acylated intermediates (Bhaskaran and Perona, 2011).

While the enzymes used for aa-tRNA-dependent modification of macromolecules recognize their substrates mainly through direct recognition of the amino acid and the nearby nucleotides of the acceptor stem on the tRNA, enzymes involved in the modification of amino acids on tRNAs may recognize a bigger surface of the tRNA including the D and/or T arms. For example, bacterial AdT (GatCAB) (Katz and Orellana, 2012a), eukaryotic and archaeal PSTK (Sherrer et al., 2008; Yuan et al., 2008) and FMT from bacteria and mitochondria of mammals (Lee et al., 1991; Varshney et al., 1991; Guillon et al., 1992; Li et al., 1996; Newton et al., 1999; Takeuchi et al., 2001) have all been shown to recognize their substrates through interactions with the acceptor and D arms. In contrast, FMT from yeast mitochondria (Vial et al., 2003) and SepCys (Fukunaga and Yokoyama, 2007; Helgadóttir et al., 2012) have been suggested to recognize tRNA through its acceptor stem and SepSecS through its acceptor and T stems (Palioura et al., 2009).

Unlike the amino acid synthesis reactions described above, the first step of tRNA-dependent tetrapyrrole synthesis in archaea, chloroplast and most bacteria involves removal of the amino acid attached to Glu-tRNA^{Glu}. In this reaction Glu is detached from tRNA^{Glu} and transferred to the enzyme glutamyl-tRNA reductase (GluTR) where it is reduced to form glutamate 1-semialdehyde (GSA). Subsequently, GSA is channeled to the second enzyme of the pathway GSA aminotransferase where it is isomerized to δ -aminolevulinic acid, which is the universal precursor for tetrapyrrole synthesis. GluTR can use Gln-tRNA^{Glu} as a substrate indicating that the enzyme is not very stringent in amino acid recognition. Additionally, experiments performed with several mutants indicated that GluTR accepts several variations in the tRNA sequence. In contrast to all the other enzymes discussed previously, GluTR does not tightly recognize the amino acid or any specific sequence features. Instead, the enzyme seems to recognize the global folding of tRNA^{Glu} mainly due to the nucleotide in position 47, which is absent in tRNA^{Gln} (O'Brian and Thöny-Meyer, 2002; Randau et al., 2004; Heinemann et al., 2008; Katz and Orellana, 2012a).

In some organisms that use these alternative pathways, specialized tRNAs have been found. For instance, in *Staphylococcus epidermidis* and apparently *Staphylococcus aureus* there are specialized tRNA^{Gly} isoacceptors that are used for peptidoglycan synthesis, but not for mRNA translation (Bumsted et al., 1968; Giannouli et al., 2009). Conversely, in some strains of *Acidithiobacillus ferrooxidans* a specialized tRNA^{Glu} used in translation, but unable to participate in tetrapyrrole synthesis, has been found (Levicán et al., 2005). In both cases such specialized tRNAs might ensure aa-tRNA availability for both pathways or alternatively prevent sudden changes in tRNA aminoacylation levels when usage by one of the alternative pathways changes. Nevertheless, neither of these hypotheses has been tested. When a tRNA is released from the constraints imposed by its participation in translation, its sequence can deviate from the canonical. As an example, all tRNA^{Gly} from *S. aureus* that apparently are specialized in peptidoglycan synthesis have an altered sequence that prevents binding to EF-Tu, including one that has deviated in structure (mainly at its D and T arms) to the degree that tRNA prediction software categorizes the sequence as belonging to a pseudogene (Giannouli et al., 2009). Another potential example of this is tRNA^{Other} in *Bacillus cereus*, which presents several deviations from canonical tRNA structure including a G2:A71 bulge and the fact that it presents a Trp anticodon whereas it is aminoacylated with Lys (Ataide et al., 2005). Although originally shown to be a substrate for aminoacylation *in vitro* (Ataide et al., 2005), this tRNA was later shown to be absent from ribosome fractions *in vivo*. Instead of participating in translation, it was shown to be part of a larger regulatory RNA, where it was proposed to represent an important structural element (Rogers et al., 2012). The role of the smaller tRNA like fragment has yet to be determined.

Hijacking of tRNA for non-synthetic processes

The information in tRNAs can be used for several “non-synthetic” roles which do not require donating an activated amino acid. Some of these roles, such as the usage of tRNAs as primers for viral genome replication (Mak and Kleiman, 1997) or the use of their genes as insertion sites in the genome for invading viruses (Hacker and Kaper, 2000; Williams, 2002) depend on the mere existence of these molecules, while others such as the use for regulatory functions take advantage of tRNA’s ability to act as a messenger for the metabolic status of the cell.

tRNA in regulatory processes

The ratio of aa-tRNA to deacylated tRNA depends on the availability of amino acids for aminoacylation and the speed of translation. Thus, the aminoacylation status of tRNA is a good indicator of the balance between the pathways that use amino acids (including translation and central metabolism) and amino acid synthesis or acquisition from the environment (Henkin and Yanofsky, 2002; Elf and Ehrenberg, 2005). In this context, it is not surprising that tRNAs have been hijacked out of their biosynthetic roles for use in regulatory processes. These regulatory systems usually require very small amounts of molecules and sometimes use only RNA. Thus, it has been proposed that such tRNA-based regulatory systems are cheap to produce and easy to evolve, and that for these reasons they might have already been in use in a probable ancestral RNA world (Henkin and Yanofsky, 2002). Furthermore, it has recently been proposed that the high specificity of aaRSs additionally

allows tRNA-based regulatory systems to be highly sensitive and substrate-specific (Bullwinkle and Ibba, 2016).

Some of the best-described regulatory mechanisms involving tRNA are the stringent response and transcription attenuation. Both take advantage of different “side effects” of changing the aa-tRNA/tRNA ratio. In the stringent response, a sudden decrease in amino acid availability allows accumulation of deacylated tRNAs that bind the ribosome where they stimulate the transfer of PP_i from ATP to either GDP or GTP by RelA/SpoT enzymes. These reactions form ppGpp and pppGpp, alarmones with pleiotropic effects on bacterial physiology including changes in metabolism of amino acids and nucleotides and regulation of synthesis of stable RNAs (rRNAs and tRNAs) (Liu et al., 2015). Under transcription attenuation, instead the state of tRNA aminoacylation can be directly sensed by riboswitches that control the formation of Rho independent transcription terminators and thus, modulate expression of genes coded by the transcript downstream of the terminator site. The riboswitch may sense tRNA aminoacylation levels by two different strategies. In one strategy, represented by the classic example of the *trp* operon in *E. coli*, a small open reading frame at the 5' leader region of the operon contains several Trp codons. Changes in the abundance of the specific aa-tRNA^{Trp} will change the speed of translation of this leader peptide modulating the time of residence of ribosomes in this area of the mRNA and controlling the formation of the terminator structure. In a second strategy, most frequently found in Gram-positive bacteria, the mRNA may present a riboswitch structure termed the T-box. Binding of the non-aminoacylated tRNA to the T-box prevents the formation of the transcription terminator, allowing transcription of the genes located downstream (Henkin and Yanofsky, 2002).

Similar to the effect of aa-tRNA abundance on the speed of translation of the leader peptide for transcription attenuation, changes in the availability of a specific aa-tRNA can also modify the speed of translation of genes that are rich in codons translated by this aa-tRNA. These genes are usually less sensitive than leader peptides and require large changes in amino acid availability that can potentially have strong effects on the abundance of aa-tRNA. Additionally, genes involved in signal transduction pathways can be extremely sensitive to small changes in protein levels (Elf and Ehrenberg, 2005; Sørensen et al., 2005; Subramaniam, Pan, et al., 2013; Subramaniam et al., 2014). As an example of this extreme sensitivity, *Bacillus subtilis* can sense decreases in environmental serine abundance through changes in the speed of translation of the *sinR* biofilm repressor and use this signal to trigger the formation of biofilms. Changes in codon usage of *sinR* produce only minor changes in the protein's cellular concentration. Nevertheless, as SinR has a cooperative behavior and is part of a negative feedback mechanism, this small change in protein concentration induces a strong change in colony morphology (Subramaniam, Deloughery, et al., 2013).

The modification of several tRNA nucleotides is also known to be involved in regulatory pathways. Usually these modified nucleotides are located in the anticodon loop, although there are some exceptions. One of the first to be identified is located at position 37 (next to the 3' end of the anticodon) of tRNAs reading codons with a 5' U (tRNA^{Tyr}, tRNA^{Trp}, tRNA^{Phe}, tRNA^{Leu}, tRNA^{Ser}, tRNA^{Cys}) from *E. coli*. Cells cultured in a medium lacking iron were found to have lost a methylthio modification (presenting an i6A nucleotide instead

of the normal hypermodified ms²i⁶A nucleotide), an alteration that induced the synthesis of enterobactin (a high affinity siderophore) and its aromatic amino acid precursors (Wettstein and Stent, 1968; Rosenberg and Gefter, 1969; Buck and Ames, 1984). Since then, several other modifications have been found to be involved in modulation of aerobic/respiratory metabolism (Buck and Ames, 1984; Persson et al., 1998; Björk et al., 1999; Nakayashiki et al., 2013), expression of virulence factors (Björk et al., 1999; Durand et al., 2000; Shippy and Fadl, 2014), the response to changes in amino acid availability (Laxman et al., 2013) or other stressful conditions (Golovina et al., 2009; Murata et al., 2011; Chan et al., 2012; Caballero et al., 2012; Dedon and Begley, 2014; Gu et al., 2014) in both bacteria and eukaryotes including mammals (Wei and Tomizawa, 2011; Gu et al., 2014; Endres et al., 2015). Additionally, high-throughput experiments have found changes of tRNA nucleotide modifications or the enzymes involved in the modification pathways related to stress conditions or diseases (Wang and He, 2014) suggesting that modification of tRNA might play a central role in regulation of gene expression.

In addition to nucleotide modifications, a series of bacterial toxins can cleave initiator or elongation tRNAs, thus inhibiting translation (Ogawa et al., 1999; Kaufmann, 2000; Winther and Gerdes, 2011; Ruhe et al., 2013; Cruz et al., 2015). This usually forms part of stress defense mechanisms, as in the case of PrrC, that inhibit protein synthesis under phage infection (Kaufmann, 2000) or some toxins from the VapC family that induce a dormancy state under other stressful conditions (Winther and Gerdes, 2011; Cruz et al., 2015). Nevertheless, other toxins have a very different ecological role and are used to attack nearby bacteria. This is the case of colicins D and E5 (Ogawa et al., 1999; Tomita et al., 2000) as well as some toxins from contact dependent growth inhibition systems such as WapA in *Bacillus subtilis* or some CDI toxins from *Burkholderia pseudomallei* (Koskiniemi et al., 2013; Ruhe et al., 2013). The reduction of functional tRNAs could have other consequences, as it has been shown that it can trigger the formation of dinucleotide second messengers (Kramer et al., 1988; Katz and Orellana, 2012b) or an increased usage of elongator tRNAs at initiation (Winther and Gerdes, 2011; Samhita et al., 2013; Shetty et al., 2015). Nevertheless, a physiological role for these phenomena in the context of tRNA fragmentation has not been studied yet. In eukaryotes, tRNA fragments are also produced. In addition to the effects derived from a decrease in tRNA concentration, in these organisms the fragments *per se* have a physiological role, binding polysomes or proteins involved in the siRNA and miRNA pathways and consequently affecting gene expression by mechanisms that are still not well understood (Raina and Ibba, 2014; Keam and Hutvagner, 2015).

tRNA mimics

The structural and functional adaptability of tRNA have allowed not only their use for alternative roles beyond protein synthesis, but also the appearance of processes that take advantage of their existence albeit not using them directly. For instance, several virus and mobile genetic elements use genes coding for tRNAs as an insertion site in genomes. These mobile elements have to include segments that mimic part of the tRNA gene sequence in order to enable recombination and to allow for reconstitution of the tRNA gene in cases where it is essential (Hacker and Kaper, 2000; Williams, 2002). Along the same lines, several molecules mimic the structure of tRNAs in order to control translation or use its

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machinery for other purposes. An impressive example of this are the tRNA-like structures (TLS) in the RNA genomes of several viruses (Dreher, 2009). A prototype of these mimics is the TLS located at the 3' end of the turnip yellow mosaic virus (TYMV) (Dreher, 2009; Colussi et al., 2014). When folded, the TLS adopts the classic L-shaped tRNA structure, although with a different topology based on idiosyncratic intramolecular interactions. Although the elements analogous to the D loop, T loop and V loop are positioned similar to tRNA, they interact in a different manner. This configuration allows the TLS to present two faces. One face of the TLS is the 'tRNA-like face', which closely mimics tRNA and achieves tRNA-like valylation efficiencies and eIF1A binding. The opposing side presents tRNA-deviating features, an upstream pseudoknot domain and the genomic RNA interacting to enable additional functionality (Dreher and Goodwin, 1998; Colussi et al., 2014). In contrast to tRNA, the unique topology and intramolecular interactions of the viral RNA also allows the TLS to unfold, possibly to allow the replication of the RNA genome (Colussi et al., 2014).

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Beside mimicry of foreign molecules (mobile elements and viruses) there are also several molecules within a cell that mimic its own tRNAs. One remarkable example is established by the participation of 2 molecules: tmRNA (small transfer-messenger RNA also known as SsrA and 10Sa RNA) and a tmRNA-specific binding protein called SmpB (small protein B). Together they mimic the upper (tmRNA) and lower (SmpB) halves of a tRNA, respectively. tmRNA owes its name to its action as both a transfer and a messenger RNA. The tmRNA-SmpB complex targets and rescues stalled ribosomes in a process known as trans-translation (Himeno et al., 2014; Keiler, 2015). This conserved translation surveillance pathway prevents accumulation of non-functional proteins from truncated mRNA (Karzai et al., 1999; Chadani et al., 2010). The tmRNA-SmpB complex mimics tRNA throughout the process. First, it is aminoacylated with alanine by alanyl-tRNA synthetase. Ala-tmRNA then enters the A-site of the stalled ribosome on a truncated mRNA to receive the nascent polypeptide from peptidyl-tRNA in the P-site. Then peptidyl-Ala-tmRNA translocates to the P-site, which exchanges the template switching the stalled ribosome from the translation of the defective mRNA to the translation of the mRNA domain of tmRNA. Translation of the tmRNA reading frame tags the defective protein to be degraded by cellular proteases (Richards et al., 2008; Giudice et al., 2014).

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In principle, any macromolecule could mimic tRNAs and in fact, even a tRNA has been found to mimic another tRNA. In this interesting case of structural mimicry between the anticodon arm of tRNA^{Asp} and the acceptor stem of tRNA^{Glu}, a paralog of glutamyl-tRNA synthetases, glutamyl-Q-tRNA^{Asp} synthetase (Glu-Q-RS, previously YadB), activates glutamate and transfers it to the queuosine at the wobble position of tRNA^{Asp} (Blaise et al., 2004; Campanacci et al., 2004; Dubois et al., 2004; Salazar et al., 2004). This is considered an hypermodification as queuosine corresponds already to a modified guanine and Glu-Q-RS was the first aaRS paralog known to catalyze this kind of reaction. Several aspects of Glu-Q-RS are unusual. Firstly, in contrast to GluRS the enzyme can activate glutamate in the absence of tRNA (Campanacci et al., 2004; Salazar et al., 2004) and transfer it to tRNA^{Asp} instead of tRNA^{Glu} (Dubois et al., 2004; Salazar et al., 2004). Secondly, it does not glutamylate tRNA^{Asp} at its 3' terminal adenosine, but instead it transfers the amino acid to a modified queuosine nucleoside, which is the first anticodon nucleoside in tRNA^{Asp} (Blaise

et al., 2004; Salazar et al., 2004). Finally, the orientation in which a tRNA interacts with Glu-Q-RS is different from that with GluRS since the tRNA needs to come close to Glu-Q-RS's active site with its anticodon loop instead of its acceptor helix (Blaise et al., 2004). The role of this hypermodification is poorly understood. It is speculated that it might reverse the effect of a single queuosine on GAU codon binding by tRNA^{ASP} Q³⁴ during translation (Blaise et al., 2005). The high K_M for glutamate of Glu-Q-RS suggests that this glutamylation might only occur when the glutamate concentration rises in the cell (Salazar et al., 2004). If this is true, the hypermodification may be a sensor, allowing Glu to affect the speed of GAU decoding during translation (Blaise et al., 2005) and, consistent with this, Glu-Q-RS seems to have a role in regulating the response to osmotic stress in *Shigella flexneri* (Caballero et al., 2012).

Similar to the example of SmpB in the mimicry of tRNA, there are several other proteins that mimic tRNA. Also similar to SmpB, all these proteins bind to the ribosome and have a function in translation. Presumably, primordial ribosomes prior to the last common ancestor catalyzed peptide-bond formation without the addition of translation factors, however these specialized factors greatly enhance the rate of translation. For instance, elongation factor G (EF-G) is a GTPase that binds the ribosome and upon GTP hydrolysis, accelerates translocation 4–5 fold (Shoji et al., 2009). EF-G is organized into 5 domains, of which domains I and II resemble in EF-Tu structure, while III–V resemble that of A-site tRNA. By virtue of structurally mimicking the tRNA-EF-Tu complex, domains III–V occupy the A-site tRNA to occlude tRNA from entering (Gao et al., 2009; Zhou et al., 2013). EF-G is not the only protein to have evolved A-site tRNA mimicry, in addition ribosome release (RRF) and recycling factors, (RF1/2, RF3) mimic tRNA and bind to similar regions. Despite these factors all binding at or near the A-site, each have functions that differ considerably. RF1/2 and RF3 bind ribosomes that have reached a stop codon to form an anticodon-codon interaction and hydrolyze the tRNA-peptidyl linkage with a conserved GQQ motif that mimics the CCA end of a tRNA (Zhou et al., 2012). RRF traverses between the A- and P-site to disassemble the ribosome into separate subunits, mediated by EF-G catalyzed translocation (Weixlbaumer et al., 2007). Therefore access to the A-site of the ribosome provides translation factors the opportunity to control each aspect of protein synthesis, however the cavity of the ribosome's entrance imposes a structural barrier that requires such proteins to mimic tRNA.

Protein-tRNA mimics are not all confined to interact with the entrance of the ribosome. Elongation factor P (EF-P) is an acidic protein comprised of three β -barrel domains, arranged in a format that notably resembles tRNA, though the domains corresponding to the anticodon arm are somewhat smaller (Hanawa-Suetsugu et al., 2004). According to biochemical studies and a co-crystal structure with the ribosome, EF-P is positioned between the P/E site, making various contacts with the peptidyl-tRNA to entropically provide the ribosome the necessary velocity when translating consecutive proline residues, as proline forms the slowest peptide bond (Blaha et al., 2009; Doerfel et al., 2013; Doerfel et al., 2015). The considerable structural similarity between EF-P and tRNA gives EF-P a distinct opportunity to interact with enzymes that tRNA may also interact with. In particular, EF-P is post-translationally modified in some organisms by a mechanism reminiscent of tRNA-aminoacylation (Navarre et al., 2010; Roy et al., 2011). The addition of (R)- β -lysine onto the

conserved lysine residue at the acceptor stem tip of EF-P augments EF-P's function by increasing the observed K_M for paused ribosomes (Doerfel et al., 2013). Interestingly, EF-P is glycosylated in some beta- and gamma-proteobacteria with a rhamnose molecule at an analogous arginine residue, calling into question how this evolutionary partnership arose while still accommodating important contacts with the ribosome (Rajkovic et al., 2015; Lassak et al., 2015).

Recent crystal and cryoEM structures revealed the energy-dependent translational throttle A (EttA) protein enters the E-site of the ribosome to regulate translation elongation in a manner dependent on the ratio of ATP/ADP (Boël et al., 2014). EttA is composed of four domains that bind ADP or ATP and a long C-terminal α -helix that interacts with the E and P-site of the ribosome. The C-terminal extension mimics tRNA to control protein synthesis directly by interacting with the peptidyl-transfer center when initiator tRNA is bound. For example, when ADP levels are high, such as in stationary phase for bacteria, EttA-ADP restricts synthesis of the first peptide bond, while in the presence of ATP, EttA marginally enhances the synthesis of the first peptide bond. This provides the first example of a protein tRNA mimic as having a regulatory function within the context of translation. Furthermore, it will be interesting to see whether EttA and EF-P accelerate peptide bond formation in a similar way.

Mimics from pathogens and parasites (such as TLS) have a strong selective pressure to maintain similitude to their host models. The hosts instead, have a strong selective pressure to change the structure of the model (in this case tRNA) or of the molecules that interact with it (in this case the ribosome or an aaRS), such that they stop recognizing the mimic and become more resistant to the pathogen/parasite (Elde et al., 2009; Elde and Malik, 2009). Nevertheless, most tRNA mimics are from within the same organism. Thus, there is strong selective pressure over both the mimic and the model for maintenance of mimicry (Katz et al., 2014).

Final remarks

Aa-tRNAs are central in translation of the genetic information, connecting cellular memory stored in nucleic acids with structural and catalytic functions performed mainly by proteins. Thus, these molecules are believed to have performed a major role in the evolution from a probable "RNA world" where RNA had both memory and catalytic functions to a world where these functions are performed mainly by specialized molecules: catalysis by proteins and memory by DNA. In this scenario, it is probable that beside tRNA, there are only a few other information molecules in living organisms that are as old and as well conserved in the three domains of life plus viruses. Although there are some differences between organisms, the fact that bacterial and eukaryal tRNAs can easily be interchanged for both aminoacylation and mRNA translation (Shafritz and Anderson, 1970; Berthelot et al., 1973; Weisblum, 1999) is evidence for how well these molecules have retained their structure. Such a long period of time has created multiple chances for nature to evolve new functions for these old molecules that carry an activated amino acid with very high specificity. As discussed in this review, several of these newer functions correspond to pathways unrelated to translation *per se*. These functions include several synthetic and regulatory pathways,

some of which might be very ancient themselves. For instance, indirect pathways for synthesis of aminoacyl-tRNA are thought to be very old, preceding the corresponding direct pathways (Woese et al., 2000; Ibba et al., 2000; Sheppard et al., 2008). The tRNA dependent synthesis of tetrapyrroles has also been proposed to be significantly older than the corresponding tRNA independent pathway (Schulze et al., 2006) maybe even predating LUCA and having arisen in the RNA world (Benner et al., 1989). Surprisingly, although many of these alternative pathways are old and usually use the same tRNAs as in translation, there are very few cases where a “cross-talk” between translation, alternative synthetic pathways and regulatory processes has been proposed. Potential cross-talk has been suggested for pathways that use large amounts of aa-tRNA, such as synthesis of peptidoglycan in gram-positive bacteria (Shepherd and Ibba, 2013a; Shepherd and Ibba, 2013b) or tetrapyrroles in organisms with high demand of them for either photosynthesis (Jahn, 1992) or oxygen consumption (Levicán et al., 2005; Levicán et al., 2007; Katz and Orellana, 2012a; Farah et al., 2014). Nevertheless, for the most part we are unaware of the effect that changes in tRNA have on most of the pathways using tRNA outside of translation. For instance, the effects of tRNA fragmentation or modification on the regulation of protein stability or tRNA dependent synthesis of amino acids are completely unknown. We expect that the mass-spec and high-throughput sequencing tools that have been developed in the last years, as well as the vast amount of genomic sequences that have been made available, will enhance research about the roles of tRNAs in regulating translation and the alternative pathways where these molecules have a central role.

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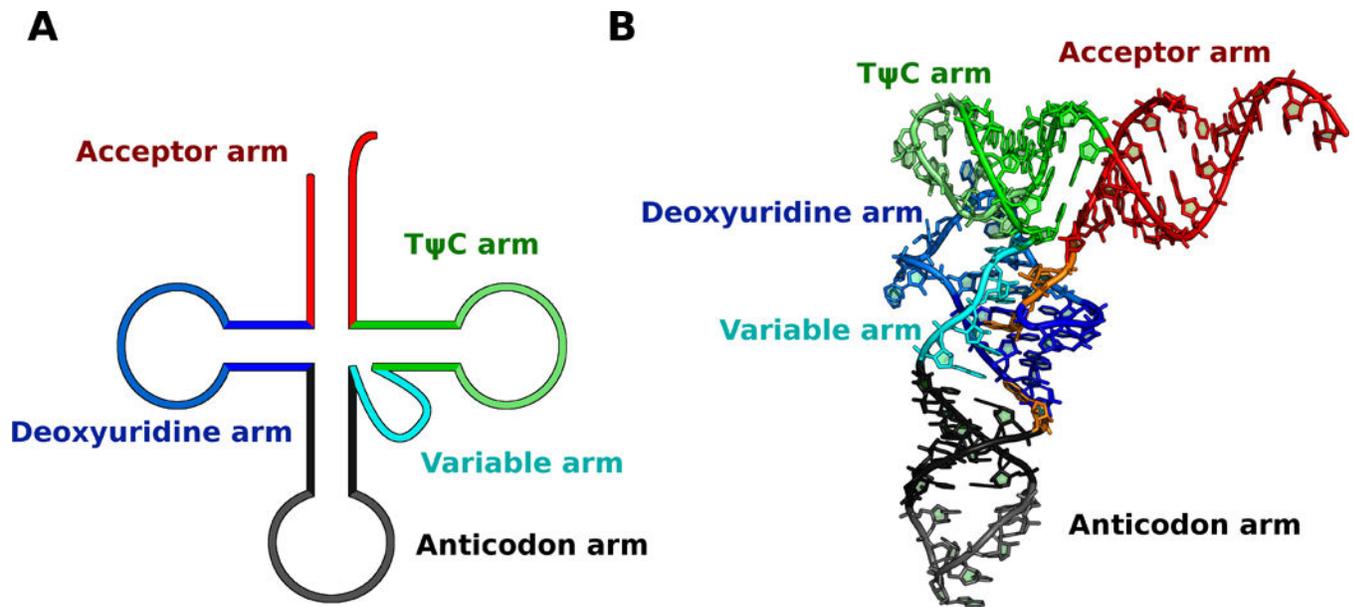


Figure 1. Structure of tRNA

Schematic representation of **A**) secondary and **B**) tertiary structure of tRNA. Tertiary structure is based on the backbone of yeast tRNA^{Asp} as in pdb model 1VTQ (Moras et al., 1980). The main parts of tRNA are highlighted in different colors, using darker colors for stems and lighter colors for loops. Used colors are **red**: acceptor stem, **blue**: deoxyuridine arm, **gray**: anticodon arm, **cyan**: variable arm, **green**: TΨC arm and **orange**: segments linking tRNA arms.

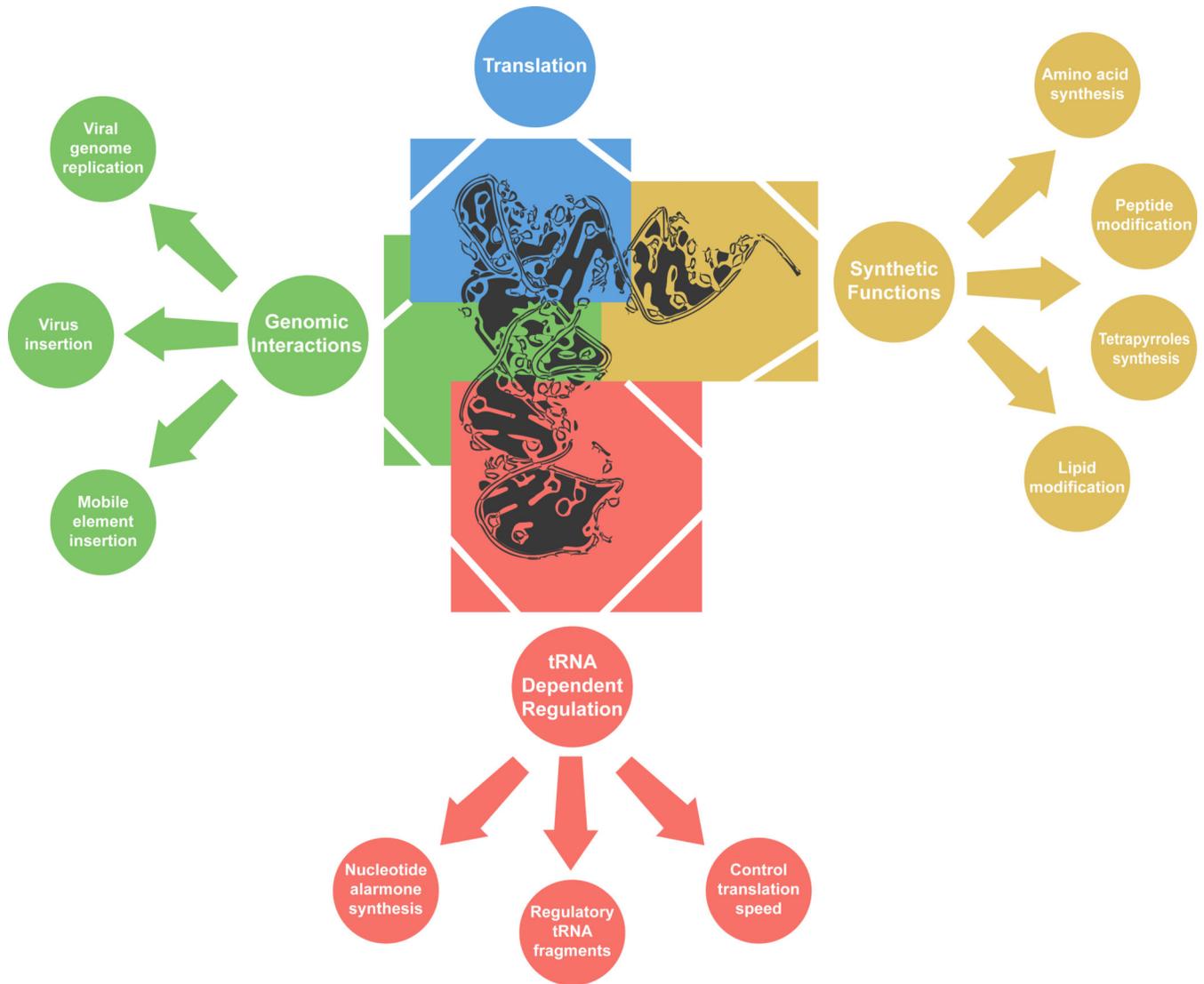


Figure 2. Biological roles of tRNA

Classification of the diverse roles that tRNAs play in cell biology. Functions of tRNAs were classified in 4 main groups, translation (blue), synthetic functions beyond translation (yellow), tRNA dependent regulation (red) and genomic interactions (green). Positioning of each group in the figure is not related to the parts of tRNA involved in specific functions.