



Mistranslation of the genetic code by a new family of bacterial transfer RNAs

Received for publication, April 5, 2023, and in revised form, May 16, 2023. Published, Papers in Press, May 22, 2023.
<https://doi.org/10.1016/j.jbc.2023.104852>

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Reviewed by members of the JBC Editorial Board. Edited by Ronald Wek

The correct coupling of amino acids with transfer RNAs (tRNAs) is vital for translating genetic information into functional proteins. Errors during this process lead to mistranslation, where a codon is translated using the wrong amino acid. While unregulated and prolonged mistranslation is often toxic, growing evidence suggests that organisms, from bacteria to humans, can induce and use mistranslation as a mechanism to overcome unfavorable environmental conditions. Most known cases of mistranslation are caused by translation factors with poor substrate specificity or when substrate discrimination is sensitive to molecular changes such as mutations or post-translational modifications. Here we report two novel families of tRNAs, encoded by bacteria from the *Streptomyces* and *Kitasatospora* genera, that adopted dual identities by integrating the anticodons AUU (for Asn) or AGU (for Thr) into the structure of a distinct proline tRNA. These tRNAs are typically encoded next to a full-length or truncated version of a distinct isoform of bacterial-type prolyl-tRNA synthetase. Using two protein reporters, we showed that these tRNAs translate asparagine and threonine codons with proline. Moreover, when expressed in *Escherichia coli*, the tRNAs cause varying growth defects due to global Asn-to-Pro and Thr-to-Pro mutations. Yet, proteome-wide substitutions of Asn with Pro induced by tRNA expression increased cell tolerance to the antibiotic carbenicillin, indicating that Pro mistranslation can be beneficial under certain conditions. Collectively, our results significantly expand the catalog of organisms known to possess dedicated mistranslation machinery and support the concept that mistranslation is a mechanism for cellular resiliency against environmental stress.

Mistranslation of the genetic code, wherein a codon is decoded with the wrong amino acid, is inherent to organisms

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from all domains of life. In basic cellular conditions, mistranslation is estimated to occur once every 10^3 – 10^4 translated codons, although environmental and metabolic factors can increase this rate (1–6). Elevated and uncontrolled mistranslation can cause irreparable damage (6–12). However, in recent years, our understanding of mistranslation has been transformed by the discoveries of cellular conditions in which mistranslation is used as a mechanism to overcome unfavorable circumstances. For instance, human cancer cells can withstand immune response challenges by translating Trp codons as Tyr (13). Similarly, global mistranslation with Met can protect mammalian and bacterial cells against oxidative stress (14, 15), while the opportunistic human pathogen *Candida albicans* mistranslates Leu codons with Ser as a mechanism to escape the host's immune response (16–19). Several other examples of mistranslation as a mechanism of cellular resiliency and survival have been described (20–29).

Aminoacyl-tRNA synthetases (aaRSs) and ribosomes are the primary sources of mistranslation. aaRSs catalyze the ligation of amino acids to tRNAs to form the aminoacyl-tRNAs, the substrates for ribosomal protein synthesis. Ribosomes and aaRSs are inherently error-prone, and their specificities are also susceptible to molecular changes (e.g., mutations or modification). Consequently, most reported cases of mistranslation are caused by the ligation of amino acids to the wrong tRNAs or incorrect pairing of aminoacyl-tRNAs with mRNA codons on the ribosomes (27, 30). However, in a few cases, dedicated mistranslation factors exist. For example, *C. albicans* encodes a unique tRNA with a dual identity. As a result, this tRNA is acylated with either Leu or Ser by the corresponding aaRSs, leading to the translation of CUG codons as either Ser or Leu (16–18).

We recently discovered a unique family of proline tRNAs in a group of *Streptomyces* bacteria that includes agriculturally important plant pathogens (31). This peculiar tRNA^{Pro} has adopted a dual identity by replacing its proline anticodon GGG with an AGC alanine anticodon (Fig. 1). To indicate its tRNA^{Pro} structure and alanine anticodon this tRNA family was named tRNA^{ProA}. tRNA^{ProA} is encoded with a distinct isoform

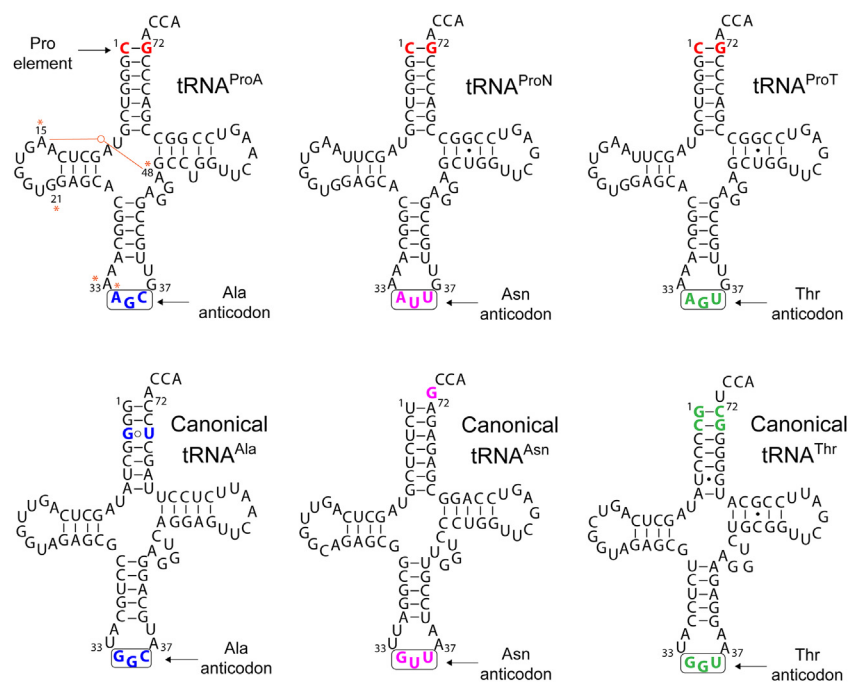


Figure 1. The predicted cloverleaf structures of the tRNA^{ProX} family. The three members of this family share the proline identity element, C1:G72 (indicated in red) but contain different anticodons. tRNA^{ProA} contains the AGC alanine anticodon (shown in blue), while the newly discovered tRNA^{ProT} and tRNA^{ProN} have AGU threonine and AUU asparagine anticodons, respectively. Members of the tRNA^{ProX} family have a combination of elements rarely found in canonical tRNAs (see Fig. S1), including G21, A33, A34, and the A15:A48 tertiary pair, which are indicated with orange asterisks in tRNA^{ProA}. The predicted secondary structures of canonical tRNA^{Ala}, tRNA^{Asn}, and tRNA^{Thr} are shown with their predicted identity elements indicated in colored and bolded fonts (32). The tRNA sequences are from *Streptomyces turgidiscabies* (tRNA^{Ala} and tRNA^{ProA}), *Streptomyces* sp. OK228 (tRNA^{ProN} and tRNA^{Asn}), and *Kitasatospora setae* KM-6054 (tRNA^{ProT} and tRNA^{Thr}).

of bacterial-type prolyl-tRNA synthetase (ProRS; called ProRSx), which appears to have coevolved with tRNA^{ProA}. When expressed recombinantly in *Escherichia coli*, tRNA^{ProA} mistranslates Ala codons with Pro, suggesting that organisms encoding tRNA^{ProA} can deliberately mistranslate their genetic code (31). tRNA^{ProA} is one of the first examples of a bacterial tRNA that evolved to mistranslate a sense codon.

In the present work, we report two new families of non-canonical tRNAs encoded by gram-positive bacteria from the *Streptomyces* and *Kitasatospora* genera. Both tRNA families have a tRNA^{ProA}-like structure but have adopted either an AUU anticodon (Asn) or an AGU anticodon (Thr) (Fig. 1). Like tRNA^{ProA}, the tRNAs are encoded next to a putative ProRSx gene, although a truncated version of ProRSx is found in some cases. We show that these novel tRNAs mistranslate their corresponding codons with Pro, leading to proteome-wide Asn-to-Pro and Thr-to-Pro changes. Pro mistranslation caused varying degrees of growth defects in *E. coli* based on the nature of the mistranslated codon. Despite the harmful impact on cell fitness, we observed that the expression of the tRNA mistranslators increased the resistance of *E. coli* to the antibiotic carbenicillin.

Results

Identification of tRNAs with dual identity

We previously determined that tRNA^{ProA} genes are annotated as alanine tRNAs (tRNA^{Ala}) in genomic databases due to automated annotation algorithms' reliance on the anticodon

sequence to establish the identity of tRNA genes. Consequently, the AGC anticodon of tRNA^{ProA} causes its annotation as tRNA^{Ala}, which hindered a complete assessment of the phylogenetic distribution of tRNA^{ProA} genes. To circumvent this, we performed searches based on sequence homology using the *Streptomyces turgidiscabies* tRNA^{ProA} as a query. The search identified 18 new genomes containing a region with a predicted tRNA gene and high homology to tRNA^{ProA}. While all the identified tRNA genes contained the signature C1:G72 base pair of tRNA^{ProA} and tRNA^{Pro}, only six tRNAs had the AGC anticodon of tRNA^{ProA}. Surprisingly, the remaining tRNA genes had either an Asn AUU (1 genome) or a Thr AGU (11 genomes) anticodon (Figs. 1 and S1). These genes are annotated as tRNA^{Asn} and tRNA^{Thr} according to their anticodon sequence. However, like tRNA^{ProA}, the tRNAs lack the known key elements for aminoacylation by their cognate aaRS (32), suggesting that they are not paired with the amino acids corresponding to their anticodon. Given the Asn or Thr anticodon combination with the Pro identity element, we named the tRNAs tRNA^{ProN} and tRNA^{ProT} (Fig. 1). Using the newly discovered tRNA^{ProN} and tRNA^{ProT} sequences as queries, we performed new searches that unearthed 21 additional organisms with tRNA^{ProA} (7), tRNA^{ProN} (1), and tRNA^{ProT} (13). Notably, tRNA^{ProN} and tRNA^{ProT} contain the same unusual and unique structural features of tRNA^{ProA} (33), including adenosine at positions 33 (A33) and 34 (A34), an A15:A48 tertiary pair, and a G21 (although several tRNA^{ProT} species contain A21) (Figs. 1 and S1). Because of the conservation of these elements, tRNA^{ProA}, tRNA^{ProN}, and tRNA^{ProT}

appear to have originated from a common ancestral tRNA and belong to a single class of noncanonical tRNAs, which we named the tRNA^{ProX} family.

A unique ProRS isoform encoded with tRNA^{ProX}

The discovery of tRNA^{ProN} and tRNA^{ProT} prompted us to examine their genomic context. We previously showed that tRNA^{ProA} is encoded next to a *proSx* gene encoding ProRSx, a distinct form of bacterial ProRS (31). Consistent with this observation, we found that every newly identified tRNA^{ProA} gene is flanked by a *proSx* gene except for *Streptomyces vinaceus*, which lacks this gene (Figs. 2A and S2). Furthermore, almost half of the bacteria that encode tRNA^{ProT} also possess a *proSx* gene (Figs. 2A and S2). However, in the other half of organisms with tRNA^{ProT} and the two encoding tRNA^{ProN}, a truncated *proSx* gene encoding roughly 200 residues is found (Figs. 2A and S2). Sequence alignments showed that the remaining section of the gene matches the C-terminal region of the ProRSx protein, which comprises the tRNA anticodon-binding domain (Figs. 2A and S3). A phylogenetic analysis of the ProRS enzymes revealed two distinct clades for ProRSx, indicating that ProRSx emerged from the bacterial-type ProRS and continued to evolve divergently (Fig. 2B). The divergence of the ProRSx enzyme family appears to be related to the identity of their accompanying tRNA^{ProX} gene as ProRSx located with the tRNA^{ProN} and tRNA^{ProT} cluster separately from ProRSx sequences encoded with tRNA^{ProA} (Fig. 2B). This suggests that ProRSx has coevolved with the corresponding tRNAs to adapt to changes in the anticodon. Thus, we surmise that the last base of the anticodon may have been the evolutionary driving force since tRNA^{ProN} and tRNA^{ProT} share U36 and tRNA^{ProA} contains C36. A multiple sequence alignment

guided by the bacterial ProRS-tRNA^{Pro} complex showed that the residues predicted to interact with N36 vary between the two ProRSx subfamilies (Fig. S3) (31).

Phylogenetic distribution and evolution of ProRSx and tRNA^{ProX}

The 46 organisms now known to encode ProRSx and tRNA^{ProX} are found in bacteria from the *Actinomycetes* class, primarily in *Streptomyces* species and a few *Kitasatospora* species (Table S1 and Fig. S1). These organisms represent a small subset of the more than 3000 *Streptomycetaceae* genomes that are publicly available. Notably, these genes are encoded in 4 of the more than 12 species known to cause plant diseases, including strains of *Streptomyces ipomoeae* (34), *S. turgidiscabies*, *Streptomyces reticuliscabiei* (35) (same genomospecies), *Streptomyces griseiscabiei* (36), and *Streptomyces caniscabiei* (37, 38). However, in several cases, ProRSx/tRNA^{ProX} is only present in one of the several available strains of these pathogens, suggesting either a recent acquisition or ancestral loss with maintenance in only one known lineage.

To better contextualize the phylogenetic distribution and evolution of ProRSx and tRNA^{ProX}, we constructed a phylogenetic tree to represent the relationship between select strains of *Streptomyces*, *Kitasatospora*, and *Streptacidiphilus* using multilocus sequence analysis (39). In addition to the 46 genomes encoding ProRSx and tRNA^{ProX}, we included 38 strains to represent the large diversity of *Streptomyces* (39), the three major clades of *Kitasatospora* (40), and *Streptacidiphilus* (41). While no available genomes of *Streptacidiphilus* contain ProRSx, the genus was included because it is sister to the *Kitasatospora* genus (40). Whether the ancestor of *Streptacidiphilus* lost ProRSx or if ancestral lineages of *Kitasatospora*

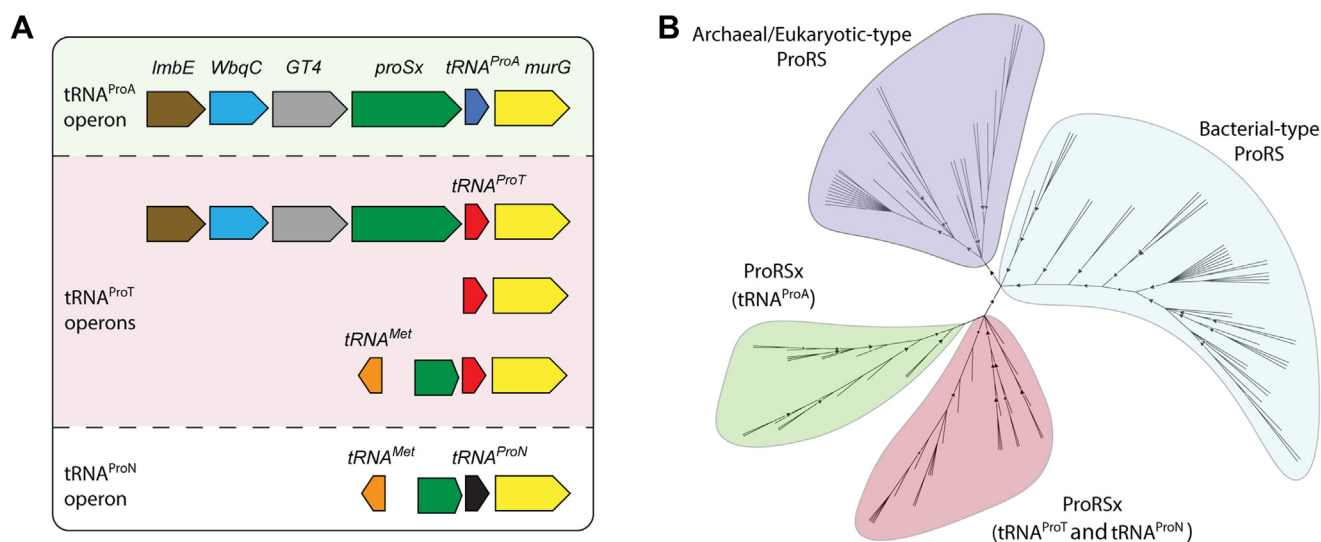


Figure 2. The tRNA^{ProX} operon. A, the predicted operon organization of the tRNA^{ProA}, tRNA^{ProT}, and tRNA^{ProN} families. All families are encoded upstream of a putative *murG* gene. tRNA^{ProA} and some tRNA^{ProT} genes are encoded downstream of a full-length *proSx* (ProRSx) gene (green), *GT4* (gray), *WbqC* (blue), and *lmbE* (brown). In some organisms, tRNA^{ProT} is encoded in an operon composed of either only tRNA^{ProT} and *murG* or a truncated *proSx* gene with a flanking methionine tRNA gene (*tRNA^{Met}*, orange). tRNA^{ProN} is only found in operons with the *tRNA^{Met}* and truncated *proSx* configuration. The complete list of operons from each organism is shown in Fig. S2. B, phylogeny of bacterial-encoded ProRS enzymes. The branches corresponding to the Bacterial-type and Archaeal/Eukaryotic-type ProRS isoforms (canonical isoforms in bacteria) are shown in the purple and light blue clusters, respectively. ProRSx sequences form a distinct cluster from the canonical ProRS enzymes. The tRNA^{ProA}-associated ProRSx sequences (green) diverge from the ProRSx encoded with tRNA^{ProT} or tRNA^{ProN} (red). Branches with bootstrap support higher than 80% are indicated by black triangles.

and *Streptomyces* independently gained ProRSx remains unknown. The analysis revealed that ProRSx and all three tRNA^{ProX} families are distributed sporadically within the *Streptomyces* genus, while ProRSx and tRNA^{ProT} are present in two of the three defined clades of *Kitasatospora* (Fig. 3). Based on its presence across multiple genera, we hypothesize that tRNA^{ProT} is the most ancestral tRNA^{ProX}, even though tRNA^{ProA} is more prevalent in *Streptomyces*. Moreover, the presence of tRNA^{ProN} in only two closely related *Streptomyces* strains indicates that it emerged more recently. Interestingly, *Streptomyces odonnellii* clustered with other strains that encode the tRNA^{ProT}, suggesting that the duplication of the tRNA^{ProT} gene is a recent event. Whether the polyphyletic distribution of ProRSx and the three members of tRNA^{ProX} results from horizontal gene transfer of the genomic locus or ancestral loss of the genes in most lineages of *Streptomycetaceae* is unknown.

tRNA^{ProT} translates Thr codons as Pro

To investigate whether tRNA^{ProT} mistranslates Thr codons with Pro, we adapted our previously developed dual fluorescence reporter consisting of a fusion of superfolder green fluorescent protein (sfGFP) and the red fluorescent mCherry in *E. coli* (Fig. 4A) (31). The reporter is based on the critical role of the residue Thr65 in forming the sfGFP fluorophore. Pro at this position prevents proper fluorophore formation,

causing a complete loss of the sfGFP fluorescence (31, 42). mCherry is used to normalize sfGFP expression. Thus, we expected that cells expressing tRNA^{ProT} and sfGFP-mCherry would display a lower sfGFP/mCherry emission ratio than cells without tRNA^{ProT} (Fig. 4B). Indeed, we observed an ~7-fold reduction in sfGFP/mCherry fluorescence emission in *E. coli* cells expressing tRNA^{ProT} (Fig. 4C), indicating that tRNA^{ProT} can incorporate Pro in response to Thr codons. Interestingly, the coexpression of tRNA^{ProT} with its cognate ProRSx only marginally increased sfGFP/mCherry fluorescence, suggesting that endogenous *E. coli* ProRS aminoacylates tRNA^{ProT}.

Mistranslation of Asn codons by tRNA^{ProN}

We next sought to test whether tRNA^{ProN} can mistranslate Asn codons with Pro. Because Asn at position 65 of sfGFP also impairs chromophore formation (42), this reporter was unsuitable for studying Asn-to-Pro mistranslation. Instead, we adopted a gain-of-function reporter based on β-lactamase activity. We previously reported that a Pro residue at position 65 of β-lactamase is essential for its catalytic activity. Consequently, cells encoding the P65A β-lactamase mutant are sensitive to the antibiotics ampicillin and carbenicillin (31). To evaluate whether Asn has a similar effect, we mutated the Pro65 codon to AAU (Asn) in β-lactamase and compared its activity with wildtype (wt) β-lactamase in *E. coli*. We observed that cells expressing the P65N β-lactamase did not grow in media containing ampicillin, whereas cells expressing the wt enzyme displayed robust growth (Fig. 5A). This result further supports that Pro65 is essential for β-lactamase and enabled us to repurpose its activity to investigate Asn-to-Pro mistranslation. We expected that mistranslation of Asn at position 65 with Pro would yield a sufficient fraction of active β-lactamase that should increase antibiotic tolerance (Fig. 5B). Thus, we expressed P65N β-lactamase in *E. coli* in the absence or presence of tRNA^{ProN} and tested cell growth on agar plates containing varying concentrations of carbenicillin. Because tRNA^{ProN} is found next to a truncated *proSx* gene, we tested the tRNA alone. As expected, *E. coli* expressing the P65N β-lactamase variant alone showed significantly impaired growth in the presence of the antibiotic (Fig. 5C). In contrast, cells expressing tRNA^{ProN} displayed a substantial tolerance to carbenicillin, even at the highest concentration tested (Fig. 5C).

To confirm the incorporation of Pro in response to Asn codons, we used an sfGFP reporter with an N-terminal extension previously developed to facilitate the detection of mistranslation events using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (43). The 26-residue extension contains a hydrophobic peptide sequence that provides reliable ionization. The sfGFP reporter was recombinantly expressed and purified from *E. coli* cells with and without tRNA^{ProN} coexpression and analyzed by LC-MS/MS. Interestingly, while no Pro misincorporation was identified at the targeted position of the N-terminal extension, Asn-to-Pro mistranslation was detected at three different positions within sfGFP corresponding to N115, N144, and N185

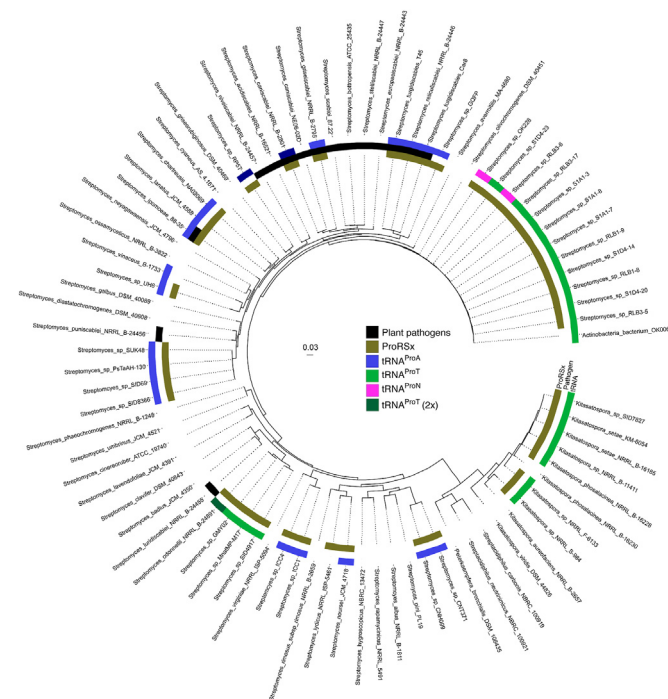


Figure 3. Phylogenetic distribution of ProRSx and tRNA^{ProX}. Maximum Likelihood phylogenetic tree constructed using multilocus sequence analysis from protein sequences of *Streptomyces* housekeeping genes *atpD*, *gyrB*, *recA*, *tpoB*, and *trpB*. Legend indicates the presence of ProRSx (brown, inner ring), pathogenicity (black, middle ring), and anticodon sequence for the corresponding tRNA^{ProX} (outer ring). The scale bar represents the phylogenetic distance of 0.03 nucleotide substitutions per site. Strains NRRL S-984 and NRRL F-6133 were renamed from *Streptomyces* to *Kitasatospora* based on this multilocus sequence analysis (MLSA).

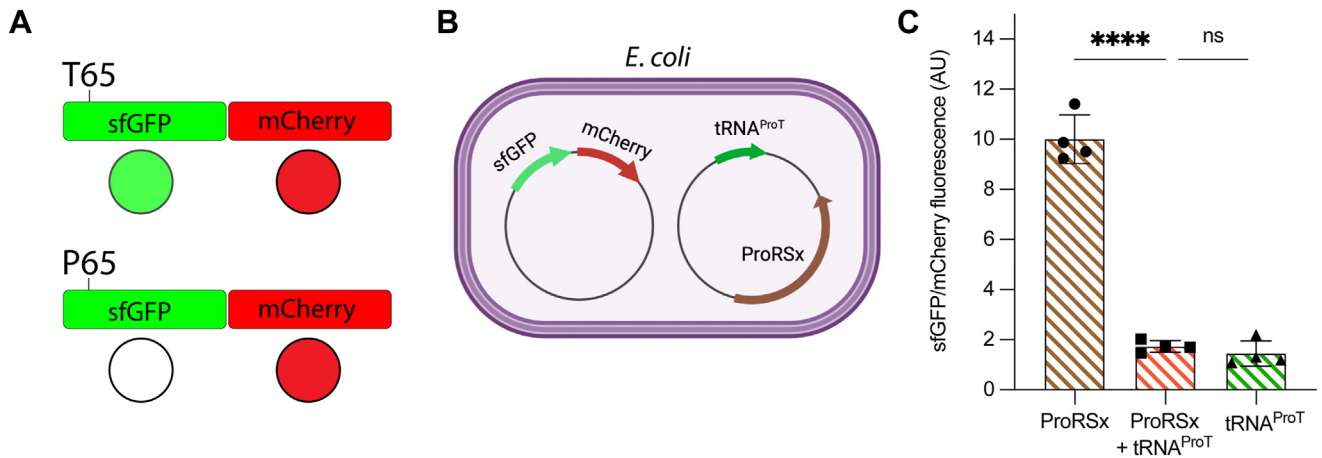


Figure 4. Detection of Thr-to-Pro mistranslation by tRNA^{ProT}. A, schematic representation of the sfGFP/mCherry fusion reporter used for monitoring *in vivo* mistranslation of Thr (ACU) codons with Pro. T65 in sfGFP is essential for fluorophore formation and fluorescence (green circle), whereas Pro at this position impairs sfGFP fluorescence resulting in nonfluorescent protein (white circle). In cells expressing tRNA^{ProT}, Thr65 can be ambiguously translated as Thr and Pro, reducing the sfGFP fluorescence relative to cells without tRNA^{ProT}. mCherry fluorescence is used to normalize the expression of sfGFP. B, *E. coli* MG1655 was transformed with plasmids encoding the sfGFP/mCherry reporter and tRNA^{ProT} (with or without ProRSx), respectively. C, the ratio of sfGFP and mCherry fluorescence at mid-exponential growth was measured in the presence of tRNA^{ProT} only (green bar), ProRSx only (brown bar), or both (orange bar). Bars represent the average of four biological replicates with the SD indicated. ns, not significant; *****p* < 0.0001 by *t* test.

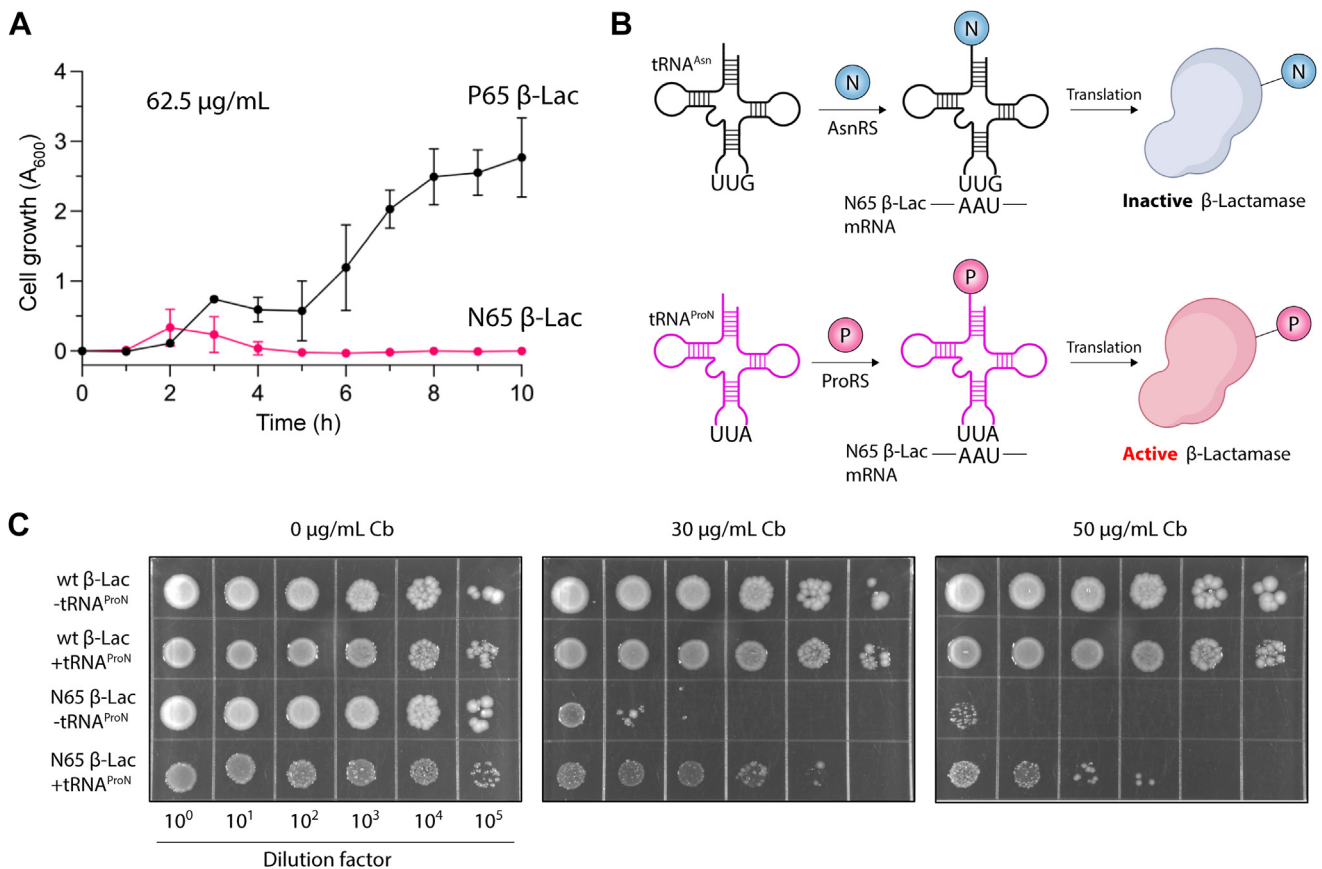


Figure 5. *In vivo* translation activity of tRNA^{ProN}. A, an N65 β -lactamase mutant is inactive and does not protect *E. coli* from β -lactam antibiotics. Cell growth (A_{600}) was monitored for *E. coli* expressing wt (P65) or N65 β -lactamase in LB medium containing ampicillin (62.5 $\mu\text{g}/\text{mL}$). Each time point is the average of three biological replicates with the SD indicated by the error bars. B, schematic representation of the β -lactamase reporter designed to monitor the translation of Asn AAU codons by tRNA^{ProN} based on the critical functional role of the P65 residue. Mistranslation of Asn AAU at position 65 with Pro by tRNA^{ProN} produces a mixed population of P65 and N65 β -lactamase, which can afford antibiotic resistance to cells. C, serial dilutions of *E. coli* cultures expressing either wt β -lactamase (wt β -Lac) or N65 β -lactamase (65N β -Lac) in the presence or absence of tRNA^{ProN} were spotted on LB-agar plates containing 0 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$, and 50 $\mu\text{g}/\text{mL}$ carbenicillin (Cb).

(Fig. S4). These substitutions were not observed in the sfGFP purified in the absence of tRNA^{ProN}. These results demonstrate that tRNA^{ProN} can mistranslate Asn codons with Pro.

Expression of tRNA^{ProX} is toxic in *E. coli*

To investigate the effect of mistranslation on cell fitness, we constitutively expressed *S. turgidiscabies* tRNA^{ProA}, *Streptomyces* sp. OK228 tRNA^{ProN}, and *Kitasatospora setae* tRNA^{ProT} in *E. coli* with or without their cognate ProRSx, except for tRNA^{ProN}, which is only encoded with a truncated ProRSx (Figs. 2A and S2). Consequently, we chose *K. setae* ProRSx (KsProRSx) for coexpression with tRNA^{ProN}, given the phylogenetic relationship between KsProRSx and the truncated *Streptomyces* sp. OK228 ProRSx (Fig. 2B). Notably, individual expression of the three tRNAs was toxic in *E. coli* cells, albeit with different degrees of severity (Fig. 6). tRNA^{ProA} caused a minor growth defect, whereas tRNA^{ProN} robustly impaired growth. In contrast, tRNA^{ProT} expression almost completely inhibited cell growth. Interestingly, cell cultures expressing tRNA^{ProT} formed white precipitated clumps, suggesting that Thr-to-Pro mistranslation causes cell wall disruption and lysis (Fig. 6C). Lastly, coexpression of the cognate *proSx* genes slightly augmented the growth defect of tRNA^{ProT} but not of tRNA^{ProA} and tRNA^{ProN}. The severity of the growth phenotypes by tRNA^{ProX} was also visible in the colony sizes of cells expressing the tRNAs after plasmid transformation. (Fig. 6C). These data suggest that the

constitutive mistranslation of Ala, Asn, and Thr codons with Pro is toxic for *E. coli*.

Aminoacylation of tRNA^{ProX} by *E. coli* ProRS

An intriguing observation from our results is that expression of ProRSx was not necessary for tRNA^{ProN}- and tRNA^{ProT}-mediated mistranslation in *E. coli* (Figs. 4 and 6), suggesting that endogenous *E. coli* ProRS aminoacylates both tRNAs. Efficient aminoacylation by *E. coli* ProRS relies on the recognition of conserved bases in the tRNA^{Pro} anticodon (G35 and G36) and acceptor stem (A73 and G72) (44, 45). All members of the tRNA^{ProX} family have A73 and G72 but contain either U or C at position 36 (Figs. 1 and S1). Moreover, tRNA^{ProT} and tRNA^{ProA} have a G35, whereas tRNA^{ProN} has a U35. We posited that the presence of these elements in tRNA^{ProX} might enable their recognition and aminoacylation by *E. coli* ProRS. To test this hypothesis, we performed *in vitro* aminoacylation assays using *E. coli* ProRS and *in vitro* tRNA transcripts. The results showed that *E. coli* ProRS has a lower aminoacylation efficiency toward all three tRNA^{ProX} relative to its cognate tRNA^{Pro} substrate (Figs. 7A and S5). However, the enzyme displayed different efficiencies toward each tRNA^{ProX}. Although tRNA^{ProT} was aminoacylated almost 4 times less than tRNA^{Pro}, it was charged ~3 and 36 times better than tRNA^{ProN} and tRNA^{ProA}, respectively. The higher aminoacylation of *E. coli* ProRS for tRNA^{ProT} may be due to the recognition of U36, as *E. coli* ProRS was shown to

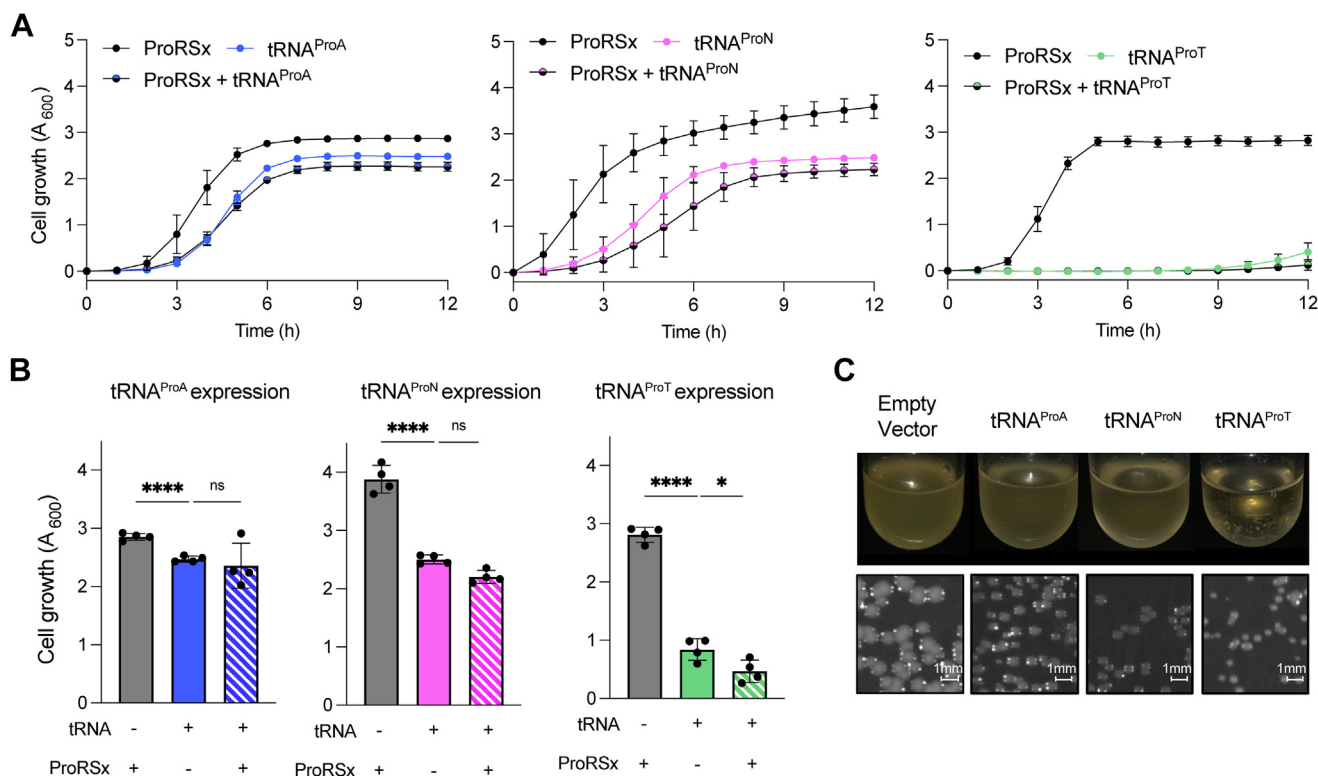


Figure 6. Toxicity of tRNA^{ProX} genes in *E. coli*. tRNA^{ProA}, tRNA^{ProN}, and tRNA^{ProT} were constitutively expressed in *E. coli* MG1655 cells in the presence and absence of cognate ProRSx. KsProRSx was coexpressed with tRNA^{ProN} because tRNA^{ProN} is found with a truncated ProRSx gene. Cell growth (A₆₀₀) was monitored for 12 h (A) or measured after 20 h (B). The plotted results represent the average of four biological replicates with the corresponding SD indicated by the error bars ns, not significant; **p* < 0.05; *****p* < 0.0001 by *t* test. C, the density of overnight *E. coli* cultures expressing empty pCDF plasmid (control) or tRNA^{ProX} genes alone is shown. Distinct colony sizes of *E. coli* cells were also observed on LB-agar plates (bottom panel).

aminoacylate a G36U tRNA^{Pro} mutant 19-fold more efficiently than a G36C mutant (Fig. 7B) (45). Next, we tested the aminoacylation level of tRNA^{ProX} in *E. coli*. Total RNA was extracted from *E. coli* expressing a tRNA^{ProX} under acidic conditions to retain the amino acid bound to tRNAs. A fraction of each tRNA^{ProX} sample was treated with a basic solution to cleave the amino acid, which allows distinguishing between aminoacylated and deacylated tRNA using specific probes for each tRNA and Northern blotting. Total RNA from *E. coli* without tRNA^{ProX} was used as a negative control. Interestingly, these assays showed that the three tRNAs are mostly aminoacylated *in vivo* (Fig. 7C). While this experiment does not reveal the amino acid attached to the tRNAs, aminoacylation by *E. coli* ProRS is likely possible, given the Pro identity elements of tRNA^{ProX}.

Pro mistranslation of Ala and Asn codons benefits *E. coli* under antibiotic stress

As shown above, mistranslation by tRNA^{ProN} and tRNA^{ProA} negatively affects cell growth under normal growth conditions (Fig. 6). However, mistranslation may offer an adaptive mechanism for survival under specific stress conditions, such as in the presence of antibiotics (28, 46, 47). To assess whether mistranslation by tRNA^{ProA} or tRNA^{ProN} is beneficial, we grew *E. coli* expressing the tRNAs with β -lactamase on LB-agar plates containing different concentrations of carbenicillin (Fig. 8A). We reasoned that, if mistranslation offers an advantage, cells expressing the mistranslating tRNA could tolerate higher antibiotic concentrations. Notably, we found that, while mistranslating cells showed a growth defect on plates without carbenicillin, they grew on plates with higher antibiotic concentrations than cells with an empty plasmid (Fig. 8B). Mistranslating cells also showed higher tolerance to ampicillin (Fig. S6). The decreased sensitivity to these β -lactam antibiotics was only observed when β -lactamase was coexpressed with the tRNAs (Fig. S6). These results provide new

evidence supporting the positive consequences of mistranslation.

Discussion

Here we described the identification and characterization of tRNA^{ProN} and tRNA^{ProT}, a new class of bacterial tRNAs, sparsely present throughout two genera of *Actinomycetota*, that emerged to mistranslate the genetic code. The discovery of tRNA^{ProN} and tRNA^{ProT} adds to the growing number of naturally occurring tRNA mistranslators reported in recent years, including several bacterial and human tRNAs (9, 48–52). The discovery of mistranslating tRNAs has been possible by re-examining sequenced genomes and reclassifying the annotated tRNA genes using a holistic analysis of each tRNA sequence and the predicted secondary structure. This approach allows the recognition of small nuances passed over by automated, high-throughput tRNA search algorithms. With the vast number of sequenced genomes, we predict that more examples of mistranslating tRNAs may be uncovered by carefully reanalyzing tRNA genes detected in these genomes.

The characterization of tRNA^{ProN} and tRNA^{ProT} in *E. coli* showed that the impact of Pro mistranslation on cell fitness depends greatly on the identity of the mistranslated codon. Thr-to-Pro mistranslation was significantly more toxic than Asn-to-Pro mistranslation. In contrast, Ala-to-Pro mistranslation was more tolerated. This difference in toxicity is remarkable because replacing residues with Pro is expected to severely affect protein structure and function (53). However, our results suggest that the substitution of Thr and Asn with Pro is more disruptive to proteins than Ala substitutions with Pro. This may be explained by the expanded catalytic and structural roles of Thr and Asn in proteins relative to the more inert function of Ala. The tRNA^{ProX} aminoacylation efficiency of *E. coli* ProRS may also contribute to the distinct cellular toxicities. The harmful effect of each tRNA correlates with the *in vitro* aminoacylation activity of *E. coli* ProRS. Thus, the

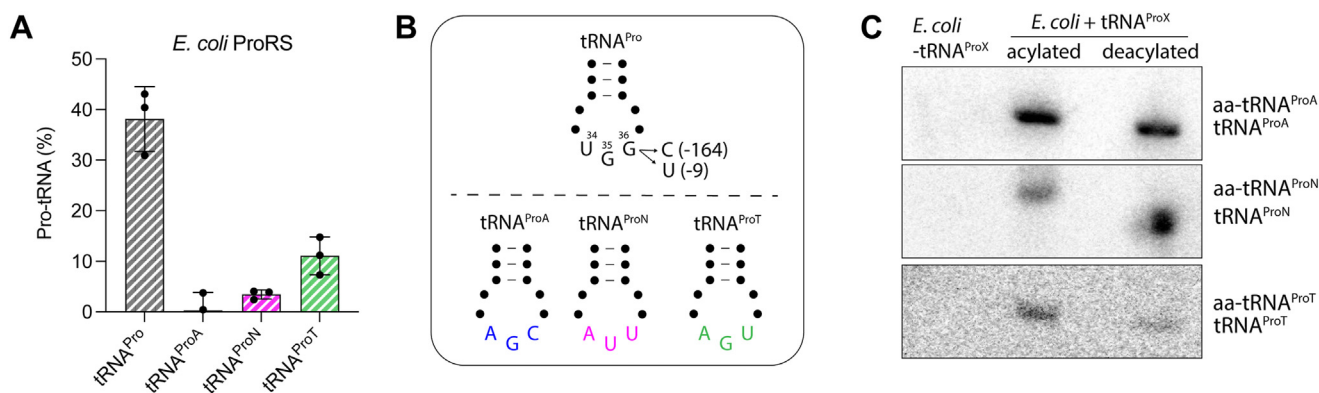


Figure 7. Aminoacylation of tRNA^{ProX} by *E. coli* ProRS. A, *in vitro* aminoacylation of tRNA^{ProA}, tRNA^{ProN}, and tRNA^{ProT} by *E. coli* ProRS. The reactions were carried out with 1 μ M ProRS, 7 μ M tRNA, and 1 mM proline at 37 $^{\circ}$ C for 30 min. Bars represent the average of three technical replicates with the SD indicated. Aminoacylation curves are shown in Fig. S5. B, anticodon stem-loop representations of tRNA^{Pro}, tRNA^{ProA}, tRNA^{ProN}, and tRNA^{ProT} are shown to highlight the difference in their anticodon sequence. Substitution of G36 to C or U in *E. coli* tRNA^{Pro} is known to decrease aminoacylation efficiency by *E. coli* ProRS by 164- and 9-fold, respectively (45). C, the steady-state aminoacylation levels of tRNA^{ProX} expressed in *E. coli* were determined by Northern blotting. Total RNA was extracted from cells expressing plasmid-borne tRNA^{ProN}, tRNA^{ProA}, or tRNA^{ProT} under acidic conditions. A fraction of the sample was then treated with NaOH to remove the amino acids from tRNAs (“deacylated”). The RNA was separated in an acid-urea gel, and tRNAs were detected by hybridization with specific radiolabeled DNA probes.

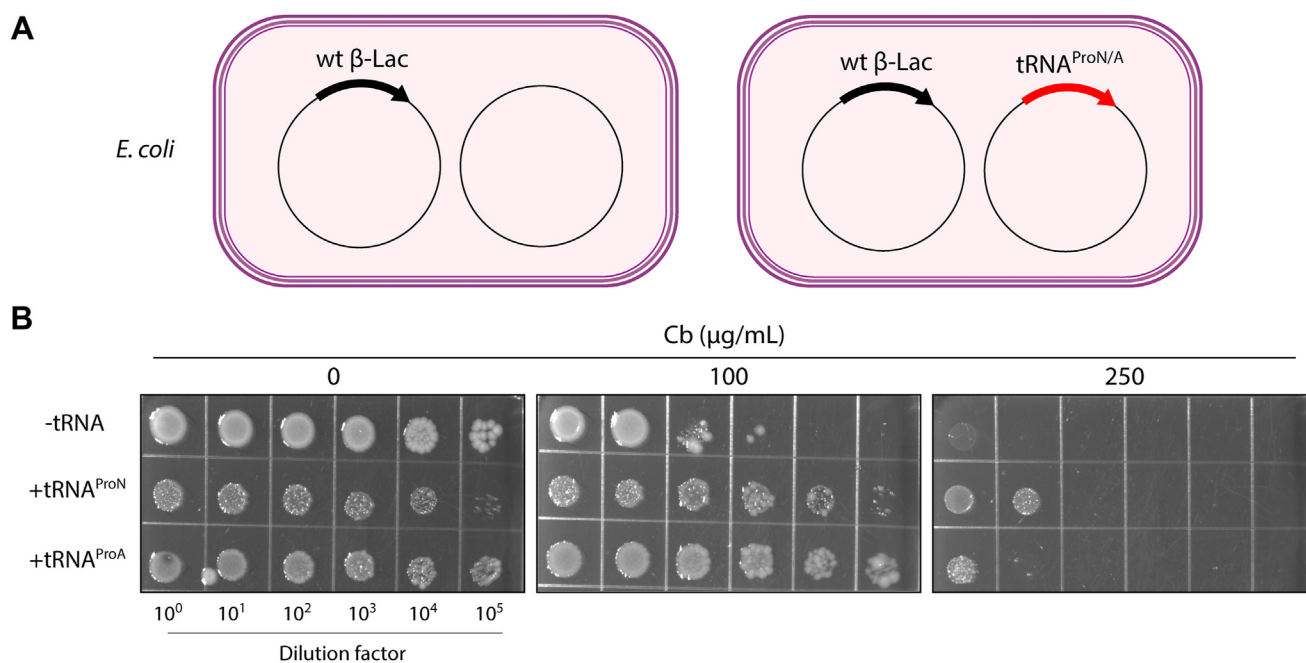


Figure 8. Increased antibiotic tolerance of *E. coli* cells expressing tRNA^{ProN} or tRNA^{ProA}. A, *E. coli* MG1655 cells harboring the plasmid encoding wt β -lactamase were transformed with a plasmid containing the tRNA^{ProN} or tRNA^{ProA} gene. B, serial dilutions of overnight cultures were spotted on LB-agar plates containing 0 μ g/ml, 100 μ g/ml, and 250 μ g/ml carbenicillin (Cb). The plates were incubated at 37 °C for 24 h.

ability of *E. coli* ProRS to aminoacylate tRNA^{ProT} more efficiently could result in higher levels of mistranslation relative to the other two tRNAs. tRNA^{ProT} could also influence Pro-tRNA^{Pro} synthesis by competing for ProRS aminoacylation with endogenous tRNA^{Pro}. We also considered the possible contribution of codon usage to the differences in toxicity. In *E. coli*, Ala codons represent 9.46% of predicted codons, whereas Thr and Asn codons comprise 5.38% and 3.92%, respectively (54). The higher Ala codon usage is inversely correlated to the severity of the observed phenotype for mistranslation of Ala codons, thus reinforcing the notion that proteins can endure Ala-to-Pro substitutions more effectively than Thr-to-Pro and Asn-to-Pro mutations. On the other hand, the mild toxicity of Asn-to-Pro relative to Thr-to-Pro may be due to the lower number of Asn codons. Different patterns of cellular toxicity have also been observed for other forms of mistranslation. Notably, *E. coli* was shown to withstand a variety of mistranslation forms such as Cys and Glu mistranslation at Pro and Gln codons, respectively (55). Recently, Gly-to-Ala mistranslation in human cells was shown to be less harmful than Ser misincorporation at Phe codons (52). In yeast, Ala mistranslation revealed codon-specific growth defects (56). Therefore, it is becoming clear that not all forms of mistranslation are equal and that the nature of mistranslated codons determines the biological impact of mistranslation, while additional factors may contribute to the distinct growth phenotypes. Lastly, the differences in tRNA^{ProX}-induced toxicity may provide clues about the phylogenetic distribution and evolution of the tRNA^{ProX} family. tRNA^{ProA}, the least toxic of the three tRNA^{ProX}, is present in more species relative to tRNA^{ProT} and tRNA^{ProN}, and it is always encoded with a full-length ProRSx (except for *S. vinaceus*). Thus, selection may act

against the more toxic tRNA^{ProT} and tRNA^{ProN}, resulting in the partial deletion or inactivation of ProRSx and a more limited phylogenetic distribution.

The specific mechanism for how Pro mistranslation of Asn and Ala codons increases carbenicillin tolerance in *E. coli* has yet to be elucidated. Interestingly, other forms of mistranslation have also been shown to decrease antibiotic sensitivity. In *E. coli*, indiscriminate mistranslation with Met protects cells from chloramphenicol (46), whereas Asp-to-Asn and Glu-to-Gln mistranslation endows *Mycobacteria* with increased resistance to rifampicin (47). The chemical diversity of these mistranslation events suggests that the randomization of the cellular protein pool may be more important than the identity of the misincorporated amino acid to decrease antibiotic susceptibility. Testing the effect of other forms of mistranslation on antibiotic resistance could provide more clues into this possibility and ultimately establish mistranslation as a bona fide mechanism of antibiotic resistance (57).

The biological function of the tRNA^{ProX} family in their host organisms is yet to be investigated. However, the increased carbenicillin tolerance of *E. coli* cells expressing tRNA^{ProN} suggests that Pro mistranslation may help tRNA^{ProX}-encoding organisms endure antibiotic exposure. It may also be possible that mistranslation offers an advantage in other environmental conditions, including in the adaptation of plant pathogenicity. However, ProRSx/tRNA^{ProX} are not collocated with known plant virulence genes. Finally, despite the tRNAs' capacity to mistranslate the genetic code in *E. coli*, their biological function in the host species may lie outside protein synthesis. tRNAs are known to have diverse functions beyond translation (58). In some cases, a particular tRNA isoacceptor can be partitioned for nonribosomal activities (59, 60). Thus, an

alternative function of tRNA^{ProX} regulated by cellular factors such as posttranslational modifications or elongation factors is possible. Further investigations will define the biological role of tRNA^{ProX} and establish the environmental conditions that promote their expression.

Experimental procedures

Identification of tRNA^{ProA} and tRNA^{ProA}-like genes

tRNA^{ProA} and tRNA^{ProA}-like genes were identified using the basic local alignment search tool (BLASTN) (61) hosted by the National Center for Biotechnology Information. The *Streptomyces turgidiscabies* tRNA^{ProA} sequence (STRTUCAR8_03649) was used as the query. Additional tRNA^{ProA} and tRNA^{ProA}-like genes were found using the “Compare Region Viewer” tool in the Bacterial and Viral Bioinformatics Resource Center (BR-BRC) (62, 63). The secondary structure and identity of the putative tRNA^{ProA} genes were predicted using tRNAscan-SE 2.0 (64). The manually aligned tRNA sequences are shown in Fig. S1.

Phylogenetic analysis and distribution of ProRSx and tRNA^{ProX}

A total of 121 sequences corresponding to ProRSx (45), Bacterial-type ProRS (42), and Archaeal/Eukaryotic-type ProRS (34) were aligned using Clustal Omega (65). The Bacterial- and Archaeal/Eukaryotic-type ProRS sequences are from the organisms encoding ProRSx (Table S1). The sequence alignment was used to build the phylogenetic tree using MEGAX (66) with the Maximum Likelihood method with 100 bootstraps. The tree was visualized with iTOL (67). Multilocus sequence analysis alignments were generated with protein sequences of housekeeping genes from *Streptomyces scabiei* 87.22 (strepdb.streptomyces.org.uk) for *atpD* (SCO5373), *gyrB* (SCO3874), *recA* (SCO5769), *rpoB* (SCO4654), and *trpB* (SCO2037) against a local database of 84 genomes using automl2 (68, 69). The 46 genomes of strains that encode ProRSx or tRNA^{ProX} were complemented with 38 additional genomes selected to represent the diversity of *Streptomyces*, *Streptacidiphilus*, and *Kitasatospora* (39–41, 70). The *S. turgidiscabies* gene sequence (STRTUCAR8_03650) was used as the query against the local sequence database with a cutoff threshold of 1e⁻¹⁰ to confirm that ProRSx was not present in the other genomes. The ML tree was constructed using default parameters for iqtree2.2 and RAxML embedded in automl2 with 1000 bootstrap iterations. The R package ggtree was used to plot the phylogeny (71) and covariates.

Bacterial strains and plasmids

E. coli Stellar cells (Takara) were used for cloning, and *E. coli* MG1655 was used for *in vivo* experiments. The HiFi DNA assembly kit (New England Biolabs) was used for molecular cloning. DNA sequencing was performed by Keck Biotechnology Resource Laboratory at Yale University to confirm plasmid sequences. The *KsProRSx* sequence was codon-optimized for expression in *E. coli* using the codon optimization tool from Integrated DNA Technologies. The

corresponding DNA was purchased from Twist Bioscience. The *S. turgidiscabies* ProRSx (*StProRSx*) gene was cloned into the pCDF vector. The *KsProRSx* was cloned into pCDF and pEVOL vectors (72). The tRNA^{ProA}, tRNA^{ProN}, and tRNA^{ProT} genes were cloned into either pCDF or pEVOL vectors under the *E. coli* constitutive promoter *proK*. A previously developed sfGFP-mCherry fusion reporter (31) was used to investigate *in vivo* mistranslation by tRNA^{ProT}. The ACC (Thr) codon at position 65 in sfGFP was mutated to ACU (Thr).

Growth conditions

E. coli strains were grown in LB medium at 37 °C and constant shaking. For selection, appropriate antibiotics carbenicillin (Cb, 100 µg/ml), spectinomycin (Spe, 100 µg/ml), kanamycin (Kan, 50 µg/ml), and chloramphenicol (Chl, 35 µg/ml) were supplemented to the medium, unless otherwise indicated.

Growth assays

Chemically competent *E. coli* MG1655 cells were transformed with the pCDF plasmid encoding a tRNA^{ProX} gene with or without a *proSx* gene (*K. setae* or *S. turgidiscabies*). After transformation, cells were recovered in S.O.C. medium (Invitrogen) at 37 °C for 1 h with constant shaking. Following recovery, transformants were plated on LB-agar with respective antibiotics and grown overnight at 37 °C. Single colonies were used to inoculate 150 µl of fresh LB medium with the corresponding antibiotics in black-sided 96-well plates with clear flat bottoms (Corning). Cell growth (absorbance, *A*) was measured at 600 nm (*A*₆₀₀) for 20 h of incubation at 37 °C and constant shaking using a BioTek synergy HTX plate reader.

Fluorescence-based assays

The pEVOL plasmid encoding tRNA^{ProT} and the sfGFP-mCherry reporter plasmid were transformed into *E. coli* MG1655 cells. Single colonies were used to inoculate LB medium containing Kan, Chl, and 0.2 mM IPTG to induce the expression of sfGFP-mCherry in a 96-well microplate. Cells were incubated for 24 h with constant shaking at 37 °C using a BioTek HTX plate reader. sfGFP (excitation 485 nm, emission 510 nm) and mCherry (excitation 587 nm, emission 610 nm) fluorescence and *A*₆₀₀ were measured. For consistency, we chose the individual mid-exponential growth phase of *E. coli* for determining sfGFP-mCherry fluorescence and mistranslation frequency. This was accomplished by determining the time at which *E. coli* cells reached the mid-exponential growth phase using the preset function “Sigmoidal, 4P, X is concentration” in Prism 9 (GraphPad). The sfGFP and mCherry fluorescence proportion was calculated at the mid-exponential growth phase.

β-Lactamase-based assays

The mistranslation capacity of tRNA^{ProN} was tested using a previously designed β-lactamase reporter (a gift from Dr Ahmed Badran, Scripps) (31). The essential Pro residue at position 65 of β-lactamase was mutated to the Asn AAU

codon. The activity of the resulting mutant was tested in *E. coli* S6020 cells. Cultures were prepared by inoculating LB medium containing Kan with single colonies and grown overnight at 37 °C. A volume of 99 μ l of fresh LB medium with Kan and 62.5 μ g/ml ampicillin was mixed with 1 μ l of overnight culture. A_{600} was monitored for 10 h at 37 °C with constant shaking using a BioTek synergy plate reader. Wildtype (wt) β -lactamase was used as a control. To test tRNA^{ProN}, the pCDF plasmid harboring tRNA^{ProN} was cotransformed with the β -lactamase (P65N or wt) reporter plasmid into *E. coli* MG1655 cells. After transformation, cells were recovered for 1 h in S.O.C. medium with shaking at 37 °C. Subsequently, cells were plated on a selective LB-agar medium and incubated overnight at 37 °C. Cell cultures were obtained by inoculating 5 ml LB medium with a single colony and incubating overnight with constant shaking at 37 °C. The overnight cultures were diluted to A_{600} of 1, which corresponded to the 10^0 dilutions, and then serially diluted to 10^1 , 10^2 , 10^3 , 10^4 , and 10^5 . A volume of 4 μ l of diluted cells was spotted on LB-agar plates with varying concentrations of carbenicillin (0 μ g/ml, 20 μ g/ml, 30 μ g/ml, 40 μ g/ml, and 50 μ g/ml). The plates were imaged after 18 to 20 h of incubation at 37 °C.

GFP purification and mass spectrometry analysis

The peptide sequence MSKGPVKVPAGVPGXGVPVGVKGGGT (43) was fused to the N terminus of sfGFP containing a C-terminal 6xHis-tag in pBAD using HiFi DNA assembly. Position X indicates the Asn AAU codon. The resulting sfGFP variant was cotransformed with pCDF with or without the tRNA^{ProN} gene into *E. coli* MG1655. Single colonies were used to inoculate LB medium containing the antibiotics Cb and Spe. Cells were grown overnight at 37 °C. The overnight culture was used to inoculate fresh LB medium with the corresponding antibiotics, and cells were grown to an A_{600} of 0.4 to 0.6. sfGFP expression was induced with 0.15% arabinose for 5 h. The suspension cultures were harvested and lysed with Bugbuster (Merck Millipore) and roll-incubated at room temperature for 20 min in a buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM Imidazole, and cComplete EDTA-free protease inhibitor mixture tablets (Roche), and 1 μ l Benzonase (Sigma-Aldrich). Lysed cells were centrifuged at 17,000g for 45 min at 4 °C. The lysate was passed through a TALON Metal Affinity Resin (Takara Bio), and the proteins were eluted with 300 mM imidazole. Subsequently, the buffer was exchanged with 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl using Amicon Ultra-4 centrifugal filter units (10,000 k, Merck Millipore) following protein concentration using the same filter units.

Samples were reduced with DTT and alkylated with iodoacetamide before digestion with trypsin and LysC. LC-MS/MS analysis was performed on a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher), equipped with a nanospray ionization source in positive ion mode with a Thermo Fisher Ultimate 3000 RSLCnano HPLC System (Thermo Fisher). Peptide mixtures were loaded into a PEPMAP100 C18 5 μ m trap column (Thermo Fisher) at

a constant flow of 30 μ l/min, holding at 60 °C. Peptides were eluted at a rate of 0.25 μ l/min and separated using a PepSep Repronil c18, 1.9 μ m, 15 cm ID 75 μ M analytical column (Bruker) throughout a 60-min gradient: 0 to 50 min, 4% - 32% acetonitrile + 0.1% formic acid; 50 to 55 min, 95% acetonitrile + 0.1% formic acid; and 55 to 60 min, 4% acetonitrile + 0.1% formic acid.

Mass spectrometry data were acquired using the data-dependent mode with a cycle time of 3 s. MS1 scan was performed in orbitrap within a range of 400 to 1600 m/z , at resolution 120,000 and autoinjection time with a standard AGC target. RF lens was set to 30%. Isolation for MS2 scans was performed in the quadrupole, with an isolation window of 0.7. MS2 scans were done in the linear ion trap at turbo scan rate, with an auto maximum injection time and a standard AGC target level. HCD was used for generating the MS2 spectrum as fixed normalized collision energy of 30%. MS2 spectra were acquired at a 15,000 resolution. All LC-MS/MS was performed at Bioinformatics Solutions Inc. in Waterloo, Ontario, Canada.

MS Raw Files were processed using PEAKS XPro (v10.6, Bioinformatics Solutions Inc). The data were searched against a custom database containing the sfGFP sequence in the *E. coli* K12 UniProt-reviewed database. Precursor ion mass error tolerance was set to 10 ppm, and fragment ion mass error tolerance was set to 0.02 Da. Semispecific cleavage with trypsin or chymotrypsin was selected with a maximum of three missed cleavages. A fixed modification of carbamidomethylation (+57.02 Da) on cysteine residues was specified. Variable modifications of deamidation (+0.98 Da) on asparagine and glutamine, as well as oxidation (+15.99 Da) on methionine, were specified. A maximum of two variable modifications per peptide was allowed. The peptide false discovery rate was set to 1% for the database search. Only confident mutations with an A-Score of 20 or higher and minimum mutation ion intensities of 1% were considered. To ensure the accuracy in identifying mutations, we applied a false discovery rate filter of <1% for peptide identification and minimum fragment ion intensity of 1% for each mutation.

Radiolabeling of oligonucleotide probes

A 50- μ l reaction mix containing 5 μ l of 10 μ M DNA oligonucleotide probes, 2.5 μ l T4-polynucleotide kinase buffer (New England Biolabs), 37.5 μ l RNase-free water, 5 μ l γ -³²P-ATP (PerkinElmer), and 2.5 μ l T4-polynucleotide kinase (New England Biolabs) was prepared and incubated for 30 min at 37 °C. Excess γ -³²P-ATP was removed with Sephadex G-25 spin columns (Cytiva). Radioactivity of the oligonucleotides was measured with a scintillation counter (Tri-Carb 3110 TR, PerkinElmer).

Northern blot analysis

E. coli MG1655 cells expressing tRNA^{ProA}, tRNA^{ProN}, or tRNA^{ProT} from a pCDF plasmid were grown overnight to inoculate fresh 20 ml LB medium with appropriate antibiotics. Cells were grown to an A_{600} of 0.4 and harvested by centrifugation at 4000g at 4 °C. The cell pellet was resuspended in

0.5 ml extraction buffer (0.3 M sodium acetate pH 4.5, 10 mM EDTA) and lysed with 0.5 ml acidic phenol (pH 4.5). Samples were vortexed for 10 s and incubated on ice for 3 min. This step was repeated 5 times. Samples were then centrifuged for 12 min at 12,000g at 4 °C. The aqueous phase containing total RNA was collected, and the sample was re-extracted with 0.25 ml extraction buffer. RNA in the aqueous supernatant was precipitated by adding 2.5 × V absolute ice-cold ethanol and incubated at –80 °C for 1 h. Samples were centrifuged for 20 min at 17,000g and 4 °C. The supernatant was discarded, and the RNA pellet was washed with 1 ml of 70% ice-cold ethanol. Centrifugation was repeated for 12 min. The supernatant was discarded, and the RNA pellet was air dried. “Deacylated tRNA” samples were resuspended in 30 µl 200 µM Tris-HCl pH 8.0 and incubated for 30 min at 37 °C, while the “acylated tRNA” samples were resuspended in 30 µl 10 mM sodium acetate pH 4.5. RNA concentrations were measured with a spectrophotometer (NanoDrop C 2000, Thermo Scientific). Total RNA from *E. coli* MG1655 was isolated as described above. One microgram of total RNA (15 µg for samples containing tRNA^{ProT}) was mixed with 2× loading buffer (0.1 M sodium acetate pH 5.0, 8 M Urea, 0.05% bromophenol blue, and 0.05% xylene cyanole). The samples were separated on a 6.5% PAGE/7 M Urea gel (Outer Plate 22.3 × 20 cm [Bio-Rad], Inner Plate 20.0 × 20.0 cm [Bio-Rad], Spacer 0.5 mm [Bio-Rad]) in 0.1 M sodium acetate pH 5.0 at 150 V for 30 min and then 250 V for 7 h at 4 °C with a PROTEAN II xi electrophoresis cell (Bio-Rad). After separation, the gel region between bromophenol blue and xylene cyanole was excised and transferred to a wet Nylon membrane (GE Healthcare) in 0.5× TBE using a Trans-Blot SD cell (Bio-Rad) at constant 25 V for 32 min. The RNA was cross-linked to the membrane in a UVC 500 Crosslinker (Amersham) and then hybridized with ³²P-labeled DNA probes targeting tRNA^{ProA} (5'-CAGG CTCCTCGGCAACG-3'), tRNA^{ProN} (5'-CGGCTCCTCGGCA GCAA-3'), and tRNA^{ProT} (5'-CAGCTCCTTGGCAACAC-3') overnight at 42 °C. The membrane was exposed against a phosphor screen for 30 min for tRNA^{ProA} and tRNA^{ProN} and overnight for tRNA^{ProT}. The screen was imaged using a phosphoimager (Typhoon, Amersham).

tRNA *in vitro* transcription and labeling

tRNAs for *in vitro* aminoacylation assays were transcribed *in vitro*. The DNA template for *in vitro* transcription was generated *via* PCR from a plasmid containing a T7 promoter. After the PCR, DNA fragments were purified with a NucleoSpin kit (Macherey-Nagel). For *in vitro* transcription, a 1-ml reaction mix of 1× transcription buffer (0.1 M Tris-HCl pH 8.0, 0.1 M MgCl₂, 2.3 M spermidine, 10% Triton, 10 mg/ml bovine serum albumin, 50 mM DTT), 4 mM ATP, 4 mM GTP, 4 mM CTP, 4 mM UTP, 10 µg DNA template, 20 mM MgCl₂, DTT 5 mM, 10 µl pyrophosphatase (Roche), and T7 RNA polymerase was incubated at 37 °C for 16 h. Subsequently, the reaction was mixed with a 0.05% bromophenol blue, 0.05% xylene cyanole solution and 50% formamide and separated with a 12% PAGE/8 M urea gel at 120 V overnight. tRNA bands were visualized under UV

light and excised from the gel. The tRNA was extracted from the gel by grinding the excised gel piece and adding 1:2 extraction buffer (0.5 M ammonium acetate and 1 mM EDTA, pH 8.0). The gel solution was incubated at 37 °C with constant shaking overnight. The gel debris was sedimented by centrifugation at 4000g for 10 min at 4 °C. The supernatant was filtered and concentrated using an Amicon Ultra (Merck) 10K filter. To precipitate the tRNA, 1 × V absolute ice-cold ethanol was added and incubated at –80 °C for 1 h. The tRNAs were precipitated by centrifugation at 13,000g for 30 min at 4 °C. The supernatant was discarded, and the tRNA pellet was washed with ice-cold 70% ethanol and centrifuged at 13,000g for 10 min at 4 °C. The supernatant was discarded, and the pellet was air dried and resuspended in 150 µl diethyl pyrocarbonate water. Subsequently, the tRNAs were labeled in a 100-µl reaction containing 50 mM glycine pH 9.0, 10 mM MgCl₂, 1 µM tRNA, 0.82 µM α-³²P-ATP (PerkinElmer), 50 µM sodium pyrophosphate, and 8.77 µg *E. coli* tRNA nucleotidyltransferase (73). The reaction was incubated for 5 min at 37 °C. A volume of 1 µl 100 µM CTP and 5 µl pyrophosphatase (Roche) were immediately added to the reaction mixture and incubated for another 2 min at 37 °C. The reaction was placed on ice, and the tRNA was extracted with 1 × V acidic phenol pH 4.6 and centrifuged at 13,000g for 2 min at 4 °C. The aqueous phase was collected and re-extracted with acidic phenol. Excess α-³²P-ATP was removed with Sephadex G-25 spin columns (Cytiva). tRNA was precipitated by adding 2 × V absolute ice-cold ethanol and 0.1 × V ammonium acetate and incubated at –20 °C overnight. The tRNA solution was centrifuged at 13,000g for 45 min at 4 °C, and the supernatant was discarded. Subsequently, the tRNA pellet was washed with 300 µl 70% ice-cold ethanol and centrifuged at 13,000g for 10 min at 4 °C. The supernatant was discarded, and the ³²P-tRNA was resuspended in 30 µl of RNase-free water.

In vitro aminoacylation assays

Aminoacylation of tRNA^{ProA}, tRNA^{ProN}, tRNA^{ProT}, and *E. coli* tRNA^{Pro} by *E. coli* ProRS was carried out in 50 mM Hepes pH 7.3, 8 mM ATP, 50 mM MgCl₂, 0.2 mg/ml bovine serum albumin, 40 mM KCl, 40 mM β-mercaptoethanol, 1 mM proline, 7 µM tRNA, 3 µl γ-³²P-tRNA, and 1 µM ProRS in a final volume of 15 µl. Reactions were incubated at 37 °C and quenched at the indicated time points by mixing 1 µl of the reaction mix with 5 µl quenching solution (200 mM sodium acetate pH 5.0 and 0.1 U/µl nuclease P1 [Sigma Aldrich]). The quenched solutions were incubated for 1 h at room temperature. A volume of 1 µl was spotted on a cellulose matrix thin layer chromatography plate (Merck), prewashed with water. The chromatography plate was run in a buffer containing 0.1 M sodium acetate pH 5.0 and 0.5% glacial acetic acid at room temperature. The plate was air dried and exposed to a phosphor screen overnight. The screen was scanned using a phosphoimager, and the AMP and Pro-AMP spots were quantified using ImageJ 1.53 (NIH).

Statistical analysis

All assays were conducted independently, and the number of biological and technical replicates is specified in the figure

legends. Statistical analyses were carried out using GraphPad Prism version 9 (GraphPad), with a two-tailed *t* test used to assess statistical significance. A *p*-value of ≤ 0.05 was considered statistically significant. The results are presented as the mean \pm standard deviation (SD). SDs are indicated as error bars.

Data availability

The mass spectrometry data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (74) partner repository with the dataset identifier PXD041335 and 10.6019/PXD041335. All the other data are contained in the article. Requests for plasmids and other materials should be sent to oscar.vargas@yale.edu.

Supporting information—This article contains supporting information.

Acknowledgments—We thank Dr Natalie Krahn for enlightening discussions and technical support and Drs Noah Reynolds and Jeffery Tharp for critically reading the manuscript.

Author contributions—D. S. and O. V.-R. conceptualization; D. B. S., K. S. H., C. R. C., and O. V.-R. methodology; D. B. S., K. S. H., and O. V.-R. validation; D. B. S. and O. V.-R. formal analysis; D. B. S., J. T. F., J. B., S. A. G., B. A. S., A. A., and O. V.-R. investigation; M. J., D. S., C. R. C., and O. V.-R. resources; D. B. S., J. T. F., S. A. G., and O. V.-R. data curation; D. B. S. and O. V.-R. writing – original draft; D. B. S., J. T. F., B. A. S., M. J., K. S. H., E. W., D. S., C. R. C., and O. V.-R. writing – review and editing; D. B. S., B. A. S., K. S. H., E. W., and O. V.-R. visualization; D. S. and O. V.-R. supervision; D. S. and O. V.-R. project administration; D. S. and O. V.-R. funding acquisition.

Funding and additional information—J. B. was, in part, supported by an educational gift from Dr Cecil B. Pickett. This work was supported by grants from the National Science Foundation (IOS-2151063 to O. V.-R.) and NIGMS, National Institutes of Health (R35GM122560-05S1 to D. S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; LC-MS/MS, liquid chromatography–tandem mass spectrometry; ProRS, prolyl-tRNA synthetase; sfGFP, superfolder green fluorescent protein.

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