Evidence for Late Resolution of the AUX Codon Box in Evolution*†‡§

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Received for publication, December 28, 2012, and in revised form, May 13, 2013. Published, JBC Papers in Press, May 21, 2013, DOI 10.1074/jbc.M112.449249

Background: Protein biosynthesis requires accurate tRNA aminoacylation.

Results: Bacterial methionyl-tRNA synthetases (MRSs) vary in their ability to reject near-cognate tRNAile transcripts containing the methionine-specifying CAU anticodon.

Conclusion: Given the degree of near-cognate discrimination among bacterial MRSs, aspects of genetic code accuracy likely were fixed relatively late in evolution.

Significance: This varied discrimination may reflect differing cellular needs for translational accuracy versus plasticity.

Recognition strategies for tRNA aminoacylation are ancient and highly conserved, having been selected very early in the evolution of the genetic code. In most cases, the trinucleotide anticodons of tRNA are important identity determinants for aminoacylation by cognate aminoacyl-tRNA synthetases. However, a degree of ambiguity exists in the recognition of certain tRNAile isoacceptors that are initially transcribed with the methionine-specifying CAU anticodon. In most organisms, the C34 wobble position in these tRNAile precursors is rapidly modified to lysidine to prevent recognition by methionyl-tRNA synthetase (MRS) and production of a chimeric Met-tRNAile that would compromise translational fidelity. In certain bacteria, however, lysidine modification is not required for MRS rejection, indicating that this recognition strategy is not universally conserved and may be relatively recent. To explore the actual distribution of lysidine-dependent tRNAile rejection by MRS, we have investigated the ability of bacterial MRSs from different clades to differentiate cognate tRNAileCAU from near-cognate tRNAileCAU.

Discrimination abilities vary greatly and appear unrelated to phylogenetic or structural features of the enzymes or sequence determinants of the tRNA. Our data indicate that tRNAile identity elements were established late and independently in different bacterial groups. We propose that the observed variation in MRS discrimination ability reflects differences in the evolution of genetic code machineries of emerging bacterial clades.

Aminoacyl-tRNA synthetases catalyze the attachment of amino acids to their cognate tRNAs for protein biosynthesis at the ribosome. Accurate recognition of cognate tRNA by each aminoacyl-tRNA synthetase is essential as tRNAs charged with noncognate amino acids may be used by the ribosome to incorrectly decode codons, introducing mutations into proteins (1). In general, there are 20 aminoacyl-tRNA synthetases, one for each of the standard amino acids (2). (Individual aminoacyl-tRNA synthetases are abbreviated using the standard one-letter amino acid abbreviation.) Methionyl-tRNA synthetase (MRS) attaches methionine to two distinct tRNA isoaccepting species: initiator tRNAileMet for decoding the initial AUG (start) codon and elongator tRNAileMet for decoding internal AUG codons, respectively. All tRNAileMet species contain a CAU anticodon. However, the single methionine codon is similar to one of the isoleucine codons (AUA). In fact, these two codons represent the only place in the genetic code where trinucleotides differing only in the type of purine in the wobble (3rd) position specify different amino acids. Solutions to this unique decoding problem vary widely across the tree of life. Higher eukaryotes use a tRNAileCAU whose U34 is post-transcriptionally modified to pseudouridine, allowing accurate decoding of AUA codons (3). Mitochondria sidestep the recognition problem by using a modified genetic code that assigns both AUA and AUG codons to methionine (4). Bacteria use a tRNAileCAU whose position 34 cytidine in the anticodon is post-transcriptionally modified by lysidine-tRNA synthase (TiIS) to lysidine (L); tRNAileCAU then specifically decodes the AUA codon (5–7). In archaea, agmatidine is introduced into this same position instead of lysidine (8, 9).

In Escherichia coli, lysidine modification switches the amino acid identity of tRNA from methionine to isoleucine and its decoding capacity from AUG methionine codons to AUA isoleucine codons (5–7). This mechanism is consistent with the prior determination that MRS from E. coli (EcMRS) belongs to an aminoacyl-tRNA synthetase group that uses cognate anticodon nucleotides as dominant identity elements for aminoacylation (10). For such synthetases, the same anticodon resi-
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dues are responsible both for aminocacylation identity and for decoding at the ribosome; in these enzymes, there is just a single code, the genetic code, which is functioning in translation.

The strength of this anticodon recognition element is illustrated by the determination that EcMRS can methionylate in vitro transcripts of the E. coli tRNAile major isoacceptor in which its GAU anticodon is changed to the methionine CAU (11). However, *Mycoplasma penetrans* MRS (MpMRS) rejects in vitro transcripts of *M. penetrans* tRNAile lacking the lysidine 34 (L34) modification, whereas EcMRS aminoacylates the *M. penetrans* tRNAile transcripts just as efficiently as its cognate tRNAileMet transcripts (12). Our results therefore point out a difference in tRNAileCAU discrimination between the *E. coli* and *M. penetrans* methionylation systems. MpMRS utilizes residues in the acceptor stem, in particular an A3-U70 base pair, to discriminate against tRNAileCAU even without its lysidine modification (12).

Loosely defined, recognition of non-anticodon tRNA residues constitutes an operational code that matches amino acid with tRNA and is distinct from the genetic code (13). As our previous study compared tRNAileCAU discrimination between just two bacterial MRS enzymes (12), we sought to determine whether strong tRNAileCAU discrimination and the use of this operational code by MpMRS are the exception or the rule for bacterial MRSs. This question is of particular interest because the operational code dictated by the acceptor stem and discriminator base of the tRNA is proposed to be the ancestral recognition mechanism for early tRNA precursors (13–15).

As the isoleucyl-tRNA synthetase and MRS catalytic sites have evolved in conjunction with tRNAileCAU sequences (16), there appear to have been some sequence motifs preferred by subtle constraints inherent in the active site of the two different enzymes, the degree of difference between tRNAileCAU and tRNAMetCAU varies significantly among species. *E. coli* and other gammaproteobacteria encode a tRNAileCAU with a C3-G70 base pair common also to its tRNAMetCAU and tRNAileMetCAU typically have C3-G70 base pairs (17). C4-G69 and C5-G68 also occur frequently in tRNAileCAU. Although these preferences are likely dictated by subtle constraints inherent in the active site of the two different enzymes, the degree of difference between tRNAileCAU and tRNAMetCAU varies significantly among species. *E. coli* and other gammaproteobacteria encode a tRNAileCAU with a C3-G70 base pair common also to its tRNAMetCAU and tRNAileMetCAU typically have C3-G70 base pairs (17). Thus the key 3-70 base pair used by MpMRS to reject tRNAileCAU is not used by EcMRS to differentiate its cognate from near-cognate tRNAileCAU (12).

Variation in the zinc binding domain among MRS enzymes might account for tRNAileCAU discrimination differences as this domain is proposed to play a role in acceptor stem recognition (18, 19). The zinc binding domain forms part of the connective polypeptide linking the halves of the Rossmann fold; depending on the organism, one or two small “knuckle” structures are present (20, 21). Strikingly, MRSs of bacterial origin have only a single knuckle, whereas MRSs of the archaeal clade have two (19, 22). Each knuckle that binds zinc typically consists of four cysteine residues in two CXXC motifs (18), although in some enzymes, the knuckle structure is generated without cysteine residues or coordination of zinc. Thus bacterial MRSs can first be divided as bacterial or archaeal in origin, and then they can be further delineated into two additional classes depending on whether they are predicted to bind one or no zinc ions (in the bacterial clade) or one or two zinc ions (in the archaeal clade) (19, 22).

In this work, we investigate the distribution of tRNAileCAU discrimination in bacterial methionylation systems. We selected seven systems from different bacterial clades to explore a variety of phylogenetic and structural characteristics. We considered MRS enzymes of eubacterial origin as well as those thought to result from archaeal gene transfer (22). These species furthermore possess a diverse set of tRNAileCAU acceptor stems. The seven enzymes also represent each of the four structural classes of MRS with respect to zinc occupancy in the connective polypeptide domain (19). Finally, the species vary with regard to the type of TiIs (tRNAileCAU lysidine synthetase) enzyme they encode: either the longer Type I TiIs with an appended tRNA binding domain or the shorter Type II TiIs (23, 24). We observe in this set of MRS enzymes a stark variation in levels of tRNAileCAU discrimination with little correlation to the structural and phylogenetic features described above. Our conclusions support the hypothesis that tRNAileCAU discrimination was established recently with respect to extant bacterial clades.

**EXPERIMENTAL PROCEDURES**

*Materials—* DNA oligonucleotides were synthesized by IDT-DNA. L-[35S]Methionine and MonoQ and HisTrap nickel columns were from Amersham Biosciences. Restriction enzymes were from New England Biolabs, and vector pCR2.1–TOPO was from Invitrogen. Expression vector pQE–70 was from Qiagen. PfuUltra DNA polymerase and XL-10 Gold were from Stratagene. *Mycoplasma penetrans* genomic DNA was a gift from Dr. Y. Sasaki of the National Institute of Infectious Diseases, Japan. Genomic DNA from *Helicobacter pylori* strain 26695 (ATCC BAA-255), *Streptococcus pneumoniae* strain R6 (ATCC BAA-255), *Bacteroides fragilis* (ATCC 25285), *Mycobacterium smegmatis* (ATCC 23037), and *Borrelia burgdorferi* strain B31 (ATCC-35210) were purchased from LGC Promochem.

*Transfer RNA Substrate Preparation—* Transfer RNAileCAU and tRNAMetCAU sequences were obtained from the Lowe tRNA database (25). tRNAs were generated by in vitro transcription of overlapping oligonucleotides (26) using T7 RNA polymerase, 5 mM NTP, 40 mM DTT, 250 mM HEPES-KOH (pH 7.5), 30 mM MgCl2, 2 mM spermidine, and 0.1 mg/ml bovine serum albumin at 37 °C for 4 h. Transcripts were separated on denaturing PAGE, eluted from the gel using an Elutrap electroelution apparatus (Schleicher & Schuell), and refolded (80 °C followed by gradual reduction of temperature in the presence of 1 mM MgCl2). Aminoacylation plateaus were used to calculate the concentration of active molecules for each tRNA preparation using 10 μl of the corresponding native enzyme (for tRNAMet samples) or EcMRS (for tRNAileCAU samples). The fraction of aminoacylatable tRNA was consistent with *E. coli* tRNAMet transcripts we typically use based on A260 quantification of tRNA (30–65% active).

*Enzyme Cloning—* MRS sequences from *S. pneumoniae* (SpMRS1, GenBank™ NP_358290.1), *H. pylori* (HpMRS, GenBank NP_207215.1), *B. fragilis* (BFMRS, GenBank YP_213853.1), *M. smegmatis* (MsMRS, GenBank YP_889680.1),
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—Enzymes from bacterial clade species H. pylori, S. pneumoniae, M. smegmatis, and M. penetrans were assayed for their ability to aminoacylate their cognate tRNA\textsubscript{Met} and near-cognate tRNA\textsubscript{ile} transcripts. The ratio of tRNA\textsubscript{Met}\textsubscript{CAU} to tRNA\textsubscript{ile}\textsubscript{CAU} methionylation initial rate was calculated for each MRS as a measure of discrimination efficiency. Enzymes displaying greater than 1000-fold difference in discrimination (as previously seen for MpMRS) were termed strong discriminators, whereas enzymes showing greater than 10-fold but less than 1000-fold difference were termed moderate discriminators. HpMRS, MsMRS, and SpMRS showed moderate discrimination, charging tRNA\textsubscript{Met}\textsubscript{CAU} more efficiently than tRNA\textsubscript{ile}\textsubscript{CAU}, by 20-, 40-, and 90-fold, respectively (Table 1). The aminoacylation profile of HpMRS provides an example of the moderately discriminating enzymes with methionylation of tRNA\textsubscript{ile}\textsubscript{CAU} observed using nanomolar enzyme concentration. This aminoacylation, whereas modest, is clearly above background and reflects multiple turnover of enzyme (Fig. 3). These results are consistent with tRNA\textsubscript{ile}\textsubscript{CAU} discrimination being widely distributed throughout the bacterial clade.

Among the enzymes tested, all bacterial-type MRSs could be classified as discriminating. Representative MRSs from major taxa including firmicutes and proteobacteria were able to reject near-cognate tRNA\textsubscript{ile}\textsubscript{CAU}. The extent of discrimination

RESULTS

Selection of MRS Enzymes—Prior phylogenetic analysis has shown that bacterial MRSs belong to two distinct clades (17). Although many bacterial MRSs appear to be of direct bacterial origin, E. coli and some other species have MRS enzymes apparently resulting from a horizontal gene transfer from archaea. Interestingly, extant archaeal MRS enzymes discriminate against a different C34 modification (agmatidine) than bacterial MRSs (lysidine). On the other hand, eukaryotic cytoplasmic
varies widely, however, as the strongly discriminating MpMRS also falls within the bacterial clade.

The zinc binding domain class does not correlate with the level of discrimination. MpMRS and HpMRS both have domains with one knuckle and one zinc ion (Fig. 2), but MpMRS is a strongly discriminating MRS with a 1600-fold difference in charging tRNACAU Met over tRNACAU Ile, whereas HpMRS shows only a moderate 20-fold level of discrimination.

Similarly, although the A3-U70 base pair of *M. penetrans* tRNACAU Ile is a key identity element for discrimination by MpMRS (12), it is not a predictor of strong discrimination by other enzymes. Like *M. penetrans*, *H. pylori* and *S. pneumoniae* encode tRNA^{le}_CAU with A3-U70 base pairs and tRNA^{Met}_CAU, with C3-G70 base pairs (supplemental Fig. S2), yet their corresponding enzymes exhibit only moderate levels of discrimination (20- and 90-fold, respectively). This result is consistent with the prior observation that EcMRS efficiently aminoacylates *M. penetrans* tRNA^{le}_CAU transcripts with the A3-U70 base pair (12); thus A3-U70 is not a universal tRNA^{le}_CAU identity element.

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**FIGURE 1. Phylogenetic distance tree of MRS sequences.** MRS of archaeal and bacterial origins are depicted in the upper and lower sections, respectively. Species tested in this work are in bold with their corresponding level of tRNACAU Ile discrimination boxed beside them. Bootstrap numbers from 1000 bootstrap replicates are depicted to provide an estimate of the robustness of the nodes. The full sequence alignment is given in supplemental Fig. S1.
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Archaeal Clade MRSs Display Great Diversity in Near-cognate Discrimination—The archaeal clade MRSs tested exhibit a striking diversity in their ability to discriminate tRNA_CAU. Initial assays with BfMRS showed no aminoacylation of tRNACAU at enzyme concentrations that catalyzed efficient aminoacylation of tRNA_CAU. As in previous work with MpMRS (12), we estimated the degree of discrimination using a higher concentration of enzyme and tRNA_CAU when compared with that used for the tRNA_CAU, sample (Fig. 4). Aminoacylation of tRNA_CAU was observed under these conditions, although multiple turnover of the enzyme was not achieved, and we estimate from initial rates of aminoacylation that BfMRS shows a 2000-fold level of discrimination.

The strong discrimination observed for BfMRS is even higher than that seen for the bacterial clade MpMRS and clearly distinct from the moderate 60-fold discrimination seen for the archaeal clade EcMRS. This was an unexpected result as BfMRS has the same two knuckle, one zinc ion configuration as EcMRS. Examination of the zinc binding domains does not suggest significant differences in the motifs or flanking sequences (Fig. 2).

Strikingly, the archaeal-type BbMRS exhibited only a 2-fold difference in aminoacylation efficiency between its cognate and near-cognate transcripts (Fig. 5). BbMRS is the only example of a nondiscriminating MRS identified in this work. This observation raises the critical question: How does a nondiscriminating MRS identified in this work. This observation raises the critical question: How does B. burgdorferi near-cognate tRNA with 2000-fold discrimination, and BbMRS is nondiscriminating (2-fold aminoacylation difference) despite their common archaeal clade assignment. B. fragilis tRNA_CAU does have an unusual U6-U67 mismatch in its acceptor stem, but the significance of this pair is unclear. The evolutionary relationship of Bacteroides to other bacteria is somewhat ambiguous, and Bacteroides species comprise their own taxon. BfMRS clusters with the MRS of Cytophaga hutchinsonii and also some archaea such as P. abyssi but is phylogenetically distant from BbMRS and EcMRS and their associated clusters (22) (Fig. 1). It is unclear whether strong tRNA_CAU discrimination among the MRS of the archaeal clade is restricted to the Bacteroides taxon or whether it is more widely distributed. Clearly, bacterial or archaeal ancestry alone does not determine tRNA_CAU discrimination ability.

DISCUSSION

The results presented in this work indicate that the extent of tRNA_CAU discrimination by MRS varies widely in bacteria and is not defined by phylogenetic position or by the structural type of the zinc binding domain. Although we showed earlier that the 3-70 base pair is an important element for MpMRS tRNA_CAU rejection, its presence or absence is not an accurate predictor of discrimination across bacteria.

Each of the bacterial clade MRSs tested to date aminoacylates its cognate tRNA_CAU at least 20-fold better than its corresponding near-cognate tRNA_CAU. In contrast, an example of nondiscrimination exists in the archaeal-type BbMRS. BbMRS clusters phylogenetically with eukaryotic MRSs that also have two

### TABLE 1

| Enzyme | Initial rate of tRNA<sub>C</sub><sup>C</sup><sub>CAU</sub> | Initial rate of tRNA<sub>C</sub><sup>C</sup><sub>CAU</sub> | Discrimination | MRS clade | Zinc knuckles | Zinc ions | tRNA<sub>C</sub><sup>C</sup><sub>CAU</sub> | % of GC | Ti<sub>C</sub><sub>EU</sub><sub>LU</sub> <sup>a</sup> |
|--------|-----------------|-----------------|----------------|-------------|-------------|----------|----------------|-------|--------------|
| BfMRS  | 2.3<sup>b</sup> | 0.46<sup>b</sup> | 2000×          | Archael      | 2           | 1        | G-C           | 43.1  | I            |
| MpMRS  | 1.5<sup>c</sup> | 1.5<sup>c</sup> | 1600×          | Bacterial     | 1           | 1        | A-U           | 25.7  | II           |
| SpMRS  | 6.0             | 0.069           | 90×            | Bacterial     | 1           | 0        | A-U           | 39.7  | I            |
| MsMRS  | 3.3             | 0.083           | 40×            | Bacterial     | 1           | 0        | G-C           | 67.4  | II           |
| EcMRS  | 18              | 0.29            | 60×            | Archaeal      | 2           | 1        | C-G           | 50.8  | I            |
| HpMRS  | 3.3             | 0.18            | 20×            | Archaeal      | 2           | 1        | A-U           | 38.9  | II           |
| BbMRS  | 3.7             | 1.9             | 2×             | Archael      | 2           | 2        | G-C           | 28.2  | I            |

<sup>a</sup> Full tRNA sequences are given in supplemental Figure S2.

<sup>b</sup> Type I TilS enzymes possess two tRNA binding domains, whereas Type II TilSs possess one tRNA binding domain.

<sup>c</sup> BfMRS was used at 40 nM to aminoacylate 2 μM cognate tRNA_CAU, and at 2 μM to aminoacylate 15.5 μM near-cognate tRNA_CAU.

<sup>d</sup> MpMRS was used at 12.5 nM to aminoacylate 2 μM cognate tRNA_CAU and at 1.55 nM to aminoacylate 20 μM near-cognate tRNA_CAU.

* Bacterial or archaeal ancestry alone does not determine tRNA_CAU discrimination ability.
zinc ions and do not require the same type of near-cognate discrimination as eukaryotes lack tRNA_{CAU}^{Ile}. However, a single case of nondiscrimination is not sufficient to define a specific relationship between the zinc binding domain class and discrimination. Additional bacterial enzymes with two zinc ions should be assayed to see whether there is indeed a correlation.

Furthermore, characterization of the full set of identity elements used by BbMRS is a priority as its cognate tRNA_{CAU}^{Met} acceptor stem contains the unusual U3-A70 pair. We have also examined the genetic background and biochemical environment of each species for putative selective pressures for robust tRNA_{CAU}^{Ile} discrimination. The need for acceptor stem-based discrimination in MpMRS could be tied to the high AT bias of the *M. penetrans* genome, one of the highest of any bacteria (33). The AUA isoleucine codon usage is only 0.4% for the *E. coli* genome but 2.1% for the *M. penetrans* genome (34). Increased usage of the AUA codon could potentially make *M. penetrans* more sensitive to occasional misreading of AUA codons by unmodified Met-tRNA_{CAU}^{Ile}, thus driving the need for a stronger MpMRS tRNA_{CAU}^{Ile} discrimination mechanism than required by *E. coli*. However, this hypothesis is not supported by the analysis of the five additional species tested here. The low GC content *B. burgdorferi* genome uses AUA codons at almost double the frequency of *M. penetrans*, but BbMRS does not discriminate (Table 1). Likewise, *M. smegmatis* is a high GC content bacteria with only one AUA codon per 1000, yet MsMRS does exhibit tRNA_{CAU}^{Ile} discrimination (34).

A selective pressure for tRNA_{CAU}^{Ile} discrimination may stem from the efficiency of the TilS enzyme in each species. MRS enzymes lacking acceptor stem-based discrimination rely on the L34 modification to avoid methionylating tRNA_{CAU}^{Ile}. Such enzymes would be expected to methionylate any unmodified tRNA_{CAU}^{Ile} present in the cytoplasm. A recent study using a temperature-sensitive *Bacillus subtilis* TilS did indeed show that loss of TilS activity was correlated with increased mistranslation of AUA codons with methionine, presumably through mismethionylation of unmodified tRNA_{CAU}^{Ile} (35). However, if TilS efficiently modifies tRNA_{CAU}^{Ile} to tRNA_{CAU}^{Met}, such a scenario would not arise, and acceptor stem-based tRNA_{CAU}^{Ile} discrimination would not be needed to prevent mischarging by MRS. Thus the discrimination disparity observed here for different MRSs may reflect in part the efficiency of TilS in each organism.

Recent work has shown that *E. coli* TilS efficiently processes pre-tRNA_{CAU}^{Ile} into tRNA_{CAU}^{Met}, blocking the opportunity for
E. coli Although the authors found no detectable level of unmodified structures of FIGURE 5.
cally, B. fragilis work do not show such a clean correlation (Table 1). Specifi-
cation of unmodified tRNACAU that a single point mutation in
4 MRS. Methionylation by 300 nM BbMRS of 4 M. penetrans transcript was only 2-fold less efficient than that of
4 M. burgdorferi tRNACAU transcript, as determined by comparing initial
rates of aminoacylation.

misacylation because pre-tRNACAU lacks a mature 3’ end (24). Although the authors found no detectable level of unmodified E. coli tRNA_\text{CAU} in vivo, it has been previously demonstrated that a single point mutation in E. coli TilS can raise the percentage of unmodified tRNA_\text{CAU} in the cell to 71% (5, 24).

A significant difference in TilS efficiency across species is plausible because there are two distinct types of TilS in bacteria. M. penetrans encodes a Type II TilS that lacks the additional tRNA binding domain found in Type I TilS (e.g. in E. coli TilS) (23, 24). Although the three species tested with the shortened Type II TilS (M. penetrans, M. smegmatis, and H. pylori) have MRS enzymes that discriminate tRNA_\text{CAU}, other results of this work do not show such a clean correlation (Table 1). Specifically, B. fragilis possesses a Type I TilS with the additional domain, but BfMRS strongly discriminates tRNA_\text{CAU}.

Although the CAU anticodon has long been considered the dominant identity element for aminoacylation by MRS, we show that the presence of this single methionine-specifying anticodon is not sufficient to confer aminoacylation in all bacterial enzymes. Of the enzymes tested, only the archaean clade BbMRS could be classified as nondiscriminating, with only 2-fold discrimination against tRNA_\text{CAU}. Even the well studied EcMRS aminoacylates its cognate tRNA 60-fold more efficiently than its near-cognate tRNA_\text{CAU}.

Certainly, the in vitro conditions used here only begin to test the ability of organisms to prevent misacylation. In addition to bacterial modification of the wobble nucleotide to lysidine by TilS, other modifications not present in the transcripts used here, codon usage, and the relative concentrations of methio-
nine versus isoleucine and tRNA_\text{Met} versus tRNA_\text{CAU} are likely to influence translational accuracy at the level of tRNA amino-
acylation. Adaptations to the decoding machinery may also enhance accuracy. For example, Mycoplasma mobile is one of several bacteria that lacks TilS; AUA codons are read by tRNA_\text{CAU}, and wobble pairing to Met AUG codons is not observed (36). This suggests that M. mobile ribosomes have adapted to ensure accuracy through a mechanism independent of known anticodon base modifications.

However, despite the numerous means by which genetic fidelity is maintained, translational accuracy may be more plastic than previously thought. Misincorporation of amino acids into the proteome may provide a cellular advantage, particularly during conditions of oxidative or other growth stress (37). In particular, methionine is attached to noncognate tRNAs at about 1% the level of tRNA_\text{Met} aminoacylation in human (HeLa) cells, and misacylation increases up to 10-fold upon viral infection or exposure to reactive oxygen species-inducing agents (38). The mediator of this misacylation in human cells is MRS, and a wide range of noncognate tRNAs is mischarged. Similarly, EcMRS exhibits in vitro mischarging of the near-cognate tRNA_\text{CAU} and tRNA_\text{CAU} in an anticodon-dependent fashion; substitutions either to the tRNA_\text{CAU}, anticodons or to the EcMRS anticodon binding domain decrease misacylation (39).

The rules governing rejection of near-cognate tRNA are clearly not universal and may reflect a relatively late evolution of discrimination strategies. In this regard, several options appear possible. For example, the final assignment of AUG codons to methionine could be, in itself, a late decision in the development of the code. This seems unlikely given the almost universal utilization of AUG as an initiator codon translated by methionine. Alternatively, a shift in the distribution of isoleucine and methionine codons may have taken place later in evolution. In this case, the current situation in mitochondria could represent the ancestral state, which, due to a universal pressure, was independently resolved into the extant structure of the AUX codon box in different clades. This possibility would explain why the strategies to ensure proper tRNA_\text{CAU} discrimination vary so widely among extant species. Alternatively or additionally, the varying degree of cognate versus near-cognate discrimination displayed by bacterial MRSs may indicate species-specific requirements for plasticity of translational fidelity and relative adaptive ability that might be enabled by low level misacylation.

Acknowledgment—We thank Tamara Hendrickson for helpful discussions and critical reading of the manuscript.
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