REPORT

Coordination of tRNA Synthetase Active Sites for Chemical Fidelity*[S]

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Background: *Mycoplasma mobile* leucyl-tRNA synthetase has lost its CP1 domain via reductive genome evolution resulting in impaired editing.
Results: Fusion of cognate and noncognate bacterial CP1 domains to the aminoacylation canonical core enhances fidelity.
Conclusion: CP1 domain insertions influence amino acid discrimination in the synthetic site.
Significance: Evolutionary addition of the CP1 domain confers multiple mechanisms to achieve fidelity.

Statistical proteomes that are naturally occurring can result from mechanisms involving aminoacyl-tRNA synthetases (aaRSs) with inactivated hydrolytic editing active sites. In one case, *Mycoplasma mobile* leucyl-tRNA synthetase (LeuRS) is uniquely missing its entire amino acid editing domain, called CP1, which is otherwise present in all known LeuRSs and also isoleucyl- and valyl-tRNA synthetases. This hydrolytic CP1 domain was fused to a synthetic core composed of a Rossmann ATP-binding fold. The fusion event splits the primary structure of the Rossmann fold into two halves. Hybrid LeuRS chimeras using *M. mobile* LeuRS as a scaffold were constructed to investigate the evolutionary protein:protein fusion of the CP1 editing domain to the Rossmann fold domain that is ubiquitously found in kinases and dehydrogenases, in addition to class I aaRSs. Significantly, these results determined that the modular construction of aaRSs and their adaptation to accommodate more stringent amino acid specificities included CP1-dependent distal effects on amino acid discrimination in the synthetic core. As increasingly sophisticated protein synthesis machinery evolved, the addition of the CP1 domain increased specificity in the synthetic site, as well as provided a hydrolytic editing site.

[S] This article contains supplemental Table 1.
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[2] The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; ProRS, prolyl-tRNA synthetase; MmLeuRS, *M. mobile* LeuRS.

link their cognate substrates via an aminoacyl bond to the correct tRNA isoacceptor (1, 2). Translation of the genetic code is set in this first step of protein synthesis when the tRNA is aminoacylated for delivery to the ribosome for messenger RNA decoding. Half of the aaRSs cannot fully distinguish between isosteric or structurally overlapping amino acid substrates and have a hydrolytic editing domain that has been fused during evolution to the ancient synthetic core of the synthetase. In these cases, the editing domain clears mistakes to maintain the high fidelity that is required for protein synthesis.

Leucyl- (LeuRS), isoleucyl- (IleRS), and valyl- (ValRS) tRNA synthetases share homologous CP1 domains that are responsible for editing by hydrolytically cleaving one or more noncognate amino acids that have been mischarged to the tRNA (2). Each of these CP1 domains is attached to the catalytic core of the enzyme via two β-strand tethers at a site that splits the primary amino acid sequence of a common Rossmann ATP-binding fold (3, 4) into two halves (5). X-ray crystal structures of LeuRS (Protein Data Bank code 1H3N), ValRS (code 1GAX), and IleRS (code 1ILE) show that the tertiary structure of the "split" Rossmann fold is intact and that the inserted CP1 editing module folds as a discrete domain (6–8).

The crystal structures of the CP1 domains of LeuRS (code 1OBC), IleRS (code 1UDZ), and ValRS (code 1WK9) with amino acid analogues have been reported (9–11). Each of the CP1 domain active sites has diverged to accommodate different amino acid specificities. The IleRS and ValRS CP1 domains hydrolyze mischarged Val-tRNA^{Ile} and Thr-tRNA^{Val}, respectively (12–15). The LeuRS CP1 domain requires a broader specificity to accommodate a number of potential synthetic active site mistakes that include methionine, isoleucine, and valine, as well as the nonstandard amino acids norvaline and norleucine (16–18).

We identified the only known example of a LeuRS, IleRS, or ValRS that is completely missing its CP1 domain (19). The gene for *Mycoplasma mobile* LeuRS (MmLeuRS) has shed the DNA encoding the editing domain of the enzyme, resulting in statistical mutations at leucine sites in the proteome. This contrasts with an artificial LeuRSΔCP1 that we constructed from *Escherichia coli* LeuRS, where the CP1 domain was deleted and maintained fidelity (20). In this latter case, the unnatural deletion mutant recovered a "pre-transfer" editing activity associated with the canonical Rossmann fold and clears mistakes by hydrolyzing misactivated aminoacyl-adenylate intermediates.

Here, we fused CP1 domains from *E. coli* LeuRS, IleRS, and ValRS to the *M. mobile* LeuRS and determined a surprising increase in amino acid discrimination in the first step of the aminoacylation reaction. This suggests that the evolutionary addition of the CP1 domain to the class I enzyme enhanced fidelity of the synthetic aminoacylation reaction in the canonical synthetic core, in addition to conferring a hydrolytic editing activity.

EXPERIMENTAL PROCEDURES

Design and Construction of Hybrid LeuRSs—Using polymerase chain reaction (PCR) mutagenesis, an SpeI restric-

tion endonuclease site (A ↓ CTAGT) was introduced into the plasmid p14bLiMmLeuRS (19) that encodes the wild type MmLeuRS gene at the sites encoding the CP1 domain fusion position of Glu-229 and Gly-232. The resultant plasmid p14bMBMmLeuRSSpeI was used to clone DNA that encoded the CP1 domain. PCR-amplified gene fragments carrying DNA sequence of the CP1 domain from *E. coli* LeuRS (p14LiMBMmLeuRS/LeuCP1H9), ValRS (p14LiMBMmLeuRS/ValCP1H1), and IleRS (p14LiMBMmLeuRS/IleCP1H11) were ligated into p14bMBMmLeuRSSpeI plasmid that was linearized with SpeI endonuclease.

Purification of Hybrid MmLeuRS—Wild type MmLeuRS and each of the His$_6$-tagged hybrid proteins were synthesized recombinantly in *E. coli* and affinity-purified using an FPLC HisTrap HP column (GE Healthcare) (20) followed by purification on a MonoQ 5/50 GL (GE Healthcare) column using low salt and high salt buffers: 20 mM Tris-HCl, pH 8.2, 5 mM NaCl and 20 mM Tris-HCl, pH 8.2, 1 M NaCl. The ion exchange column purification was followed by purification on a size exclusion Superdex 200 column (GE Healthcare) in 10 mM Tris-HCl, pH 8.0, 20 mM NaH$_2$PO$_4$, 100 mM NaCl, 5% glycerol to ensure that residual *E. coli* LeuRS, IleRS, and ValRS were removed prior to experiments.

Rapid Quench Kinetics—Multiple turnover kinetic reactions containing 5 μM protein, 12.5 μM *M. mobile* tRNA$^{Leu}_{UAA}$ (tRNA^{Leu}) transcript, and 50 μM [^{14}C]leucine (318 mCi/mmol) were carried out as described (21, 22). Single turnover kinetic reactions contained 2 μM tRNA^{Leu} and 10 μM wild type or hybrid LeuRSs complexed with leucyl-adenylate intermediate and fitted into a single exponential equation to measure k_{trans} (21, 22).

Transcription and Misaminoacylation of M. mobile tRNA^{Leu}—*M. mobile* tRNA^{Leu} was *in vitro* transcribed (19, 23) and charged with [^{14}C]isoleucine or [^{35}S]methionine using T252Y *E. coli* LeuRS editing-defective mutant (18).

Pyrophosphate Exchange—In a pyrophosphate exchange assay (20, 24), K_m and k_{cat} for activation of leucine and also isoleucine, methionine, and valine were measured using equivalent 1 mM concentrations of amino acid, ATP, and [^{32}P]PP$_i$ (900 mCi/mmol) at 30 °C. Pyrophosphate and ATP were separated by thin-layer chromatography and quantitated by phosphorimaging.

RESULTS

We used *M. mobile* LeuRS as a scaffold to fuse CP1 domains to the catalytic class I aaRS core and reconstruct an additional hydrolytic editing step that was introduced during evolution to increase fidelity for protein synthesis. We created hybrid *M. mobile* LeuRS proteins that contained the CP1 domain from *E. coli* LeuRS (MmLeuRS/CP1^{Leu}) and also the IleRS (MmLeuRS/CP1^{Ile}) and ValRS (MmLeuRS/CP1^{Val}) CP1 domains (Fig. 1). In each case, we retained the β-strand linkers that are native to the CP1 domain that was introduced. The N- and C-terminal β-strands of the CP1 domains were fused to the Rossmann fold of *M. mobile* LeuRS at Glu-229 and Gly-232 (•••^{224}WIGKEEIDG^{232}•••), respectively. This corresponds to Glu-228 and Ala-362, the naturally occurring *E. coli* LeuRS
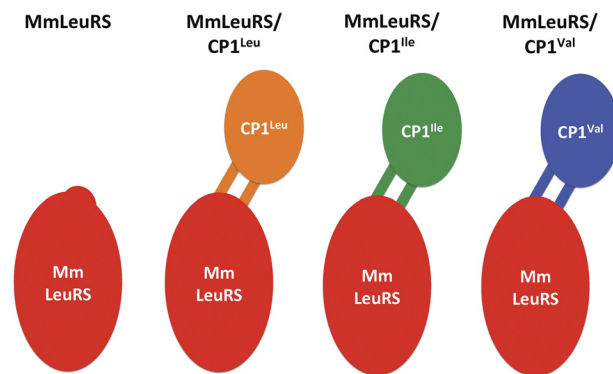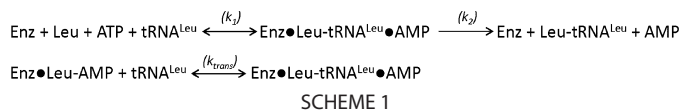

FIGURE 1. **Schematic of *M. mobile* LeuRS and CP1 hybrid mutants.** The canonical synthetic core of MmLeuRS is shown in *red*. The fused CP1 domains and their respective β-strands from *E. coli* LeuRS (CP1^{Leu}), IleRS (CP1^{Ile}), and ValRS (CP1^{Val}) are shown in *orange*, *green*, and *blue*, respectively.

TABLE 1

Multiple and single turnover kinetic measurements during tRNA aminoacylation with leucine

Methods are described in Hellmann and Martinis (21). Kinetic constants can be defined in Scheme 1.

	k_1	k_2	k_{trans}
		s^{-1}	
MmLeuRS	18.5 ± 3.4	2.1 ± 0.11	15.2 ± 2.1
MmLeuRS/CP1^{Leu}	9.7 ± 1	1.37 ± 0.12	12.6 ± 3.7
MmLeuRS/CP1^{Ile}	10.6 ± 0.13	1.67 ± 0.13	14.8 ± 1.1
MmLeuRS/CP1^{Val}	10.7 ± 0.23	0.42 ± 0.05	10.5 ± 1.1

$$\text{Enz + Leu + ATP + tRNA}^{Leu} \xrightarrow{(k_1)} \text{Enz•Leu-tRNA}^{Leu}\text{•AMP} \xrightarrow{(k_2)} \text{Enz + Leu-tRNA}^{Leu} + \text{AMP}$$

$$\text{Enz•Leu-AMP + tRNA}^{Leu} \underset{}{\overset{(k_{trans})}{\rightleftharpoons}} \text{Enz•Leu-tRNA}^{Leu}\text{•AMP}$$

SCHEME 1

fusion sites that link the flexible β-strands of the CP1 domain to the Rossmann fold (19, 25).

Using rapid quench kinetics, we compared the aminoacylation properties of the wild type *M. mobile* LeuRS that is missing the CP1 domain with each of the hybrid constructs that contained the LeuRS core fused to an *E. coli* CP1 domain from LeuRS, IleRS, or ValRS. Similar to other class I aaRSs including *E. coli* LeuRS, the multiple turnover kinetic profile was biphasic (21, 26) for the wild type *M. mobile* LeuRS and its hybrid constructs (Table 1).

The rate constant k_2 correlates to a rate-limiting product release step for charged tRNA (21, 26). The addition of the CP1 domain from either LeuRS or IleRS only slightly lowered k_2. The largest decrease was 5-fold for the LeuRS hybrid that contained CP1^{Val}, suggesting that charged Leu-tRNA^{Leu} product release was slowed further by the addition of the CP1 domain. Because the "exit complex" of LeuRS has been proposed to have the 3′-charged end of the tRNA positioned near the hydrolytic active site of the CP1 editing domain (27), it is possible that either the noncognate mismatch between CP1^{Val} and tRNA^{Leu} or the charged leucine amino acid affects the mechanism of product release. The faster rate constant, k_1, measured during multiple turnover kinetics, was decreased by less than 2-fold (Table 1) for each of the hybrid LeuRSs as compared with the wild type *M. mobile* LeuRS that is missing its CP1 domain.

The k_{trans}, measured during single turnover kinetic analysis, represents the kinetic rate constant for the transfer of the amino acid from the adenylate intermediate to tRNA^{Leu} and
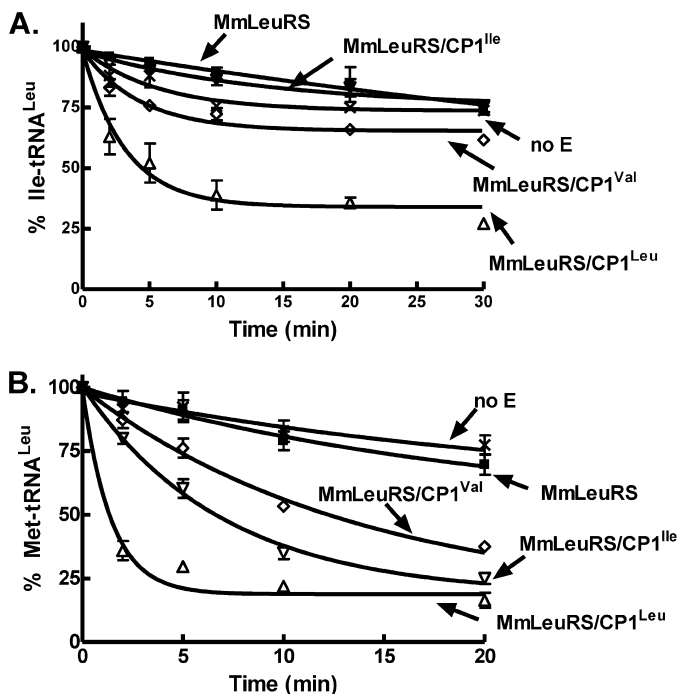
FIGURE 2. **Deacylation of charged tRNA by *M. mobile* LeuRS and hybrid mutants.** *A* and *B*, deacylation of 2 μM misaminoacylated Ile-tRNA^{Leu} (*A*) or Met-tRNA^{Leu} (*B*) by 1 μM *M. mobile* LeuRS wild type or hybrid proteins that contain an *E. coli* CP1 domain. Symbols used are as follows: ×, nonenzymatic tRNA deacylation (*no E*); ■, MmLeuRS (wild type); △, MmLeuRS/CP1^{Leu}; ▽, MmLeuRS/CP1^{Ile}; ◇, MmLeuRS/CP1^{Val}. *Error bars* represent the S.D. values based on three separate experiments.

FIGURE 3. **Bar graph representing amino acid discrimination factors relative to leucine.** Data for *M. mobile* LeuRS are shown in red, whereas data for hybrid proteins MmLeuRS/CP1^{Leu}, MmLeuRS/CP1^{Ile}, and MmLeuRS/CP1^{Val} are shown in *orange*, *green*, and *blue*, respectively. This graph is based on the measurements listed in supplemental Table S1.

correlated to other class I aaRSs (26). As would be expected as compared with other class I aaRSs (21, 26), k_{trans} was most similar to k_1 for the wild type *M. mobile* LeuRS and hybrid constructs. Significantly, this supports that the integrity of the synthetic aminoacylation site is maintained despite deep cuts into the Rossmann fold to introduce CP1 domains from different enzymes with distinct specificities.

We screened each of the hybrid LeuRS tRNA deacylation activities, which would be reminiscent of a canonical LeuRS post-transfer editing activity that hydrolyzes noncognate amino acids from tRNA. The hybrid composed of the LeuRS CP1 domain fused to the *M. mobile* LeuRS aminoacylation core (MmLeuRS/CP1^{Leu}) was faithful to its cognate specificities that ensure fidelity in producing Leu-tRNA^{Leu}. In particular, it hydrolyzed Ile-tRNA^{Leu} and Met-tRNA^{Leu} with a k_{obs} of 0.31 and 0.71 min^{-1}, respectively (Fig. 2). In comparison, the wild type *E. coli* LeuRS respectively deacylated Ile-tRNA^{Leu} and Met-tRNA^{Leu} with a k_{obs} of 1.43 and 0.69 min^{-1}. This is similar to natural LeuRSs, which have evolved to edit aliphatic standard amino acids that threaten protein synthesis fidelity (16, 18, 23, 28).

Both hybrid MmLeuRS/CP1^{Val} and MmLeuRS/CP1^{Ile} hydrolyzed Met-tRNA^{Leu} (Fig. 2*B*), with a k_{obs} of 0.07 and 0.14 min^{-1}, respectively. In comparison, the wild type *E. coli* IleRS hydrolyzed Met-tRNA^{Ile} with a k_{obs} rate of 1.32 min^{-1}. The flexibility of the unbranched aliphatic side chain of methionine likely facilitates binding in the hydrolytic editing active site similar to full-length aaRSs. Consistent with the robustness and the specificities of the CP1 hydrolytic activities of the hybrid
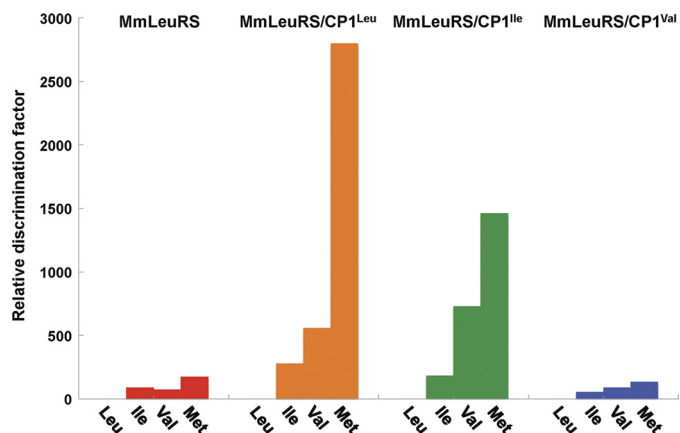
M. mobile LeuRSs, mischarging did not occur for MmLeuRS/CP1^{Leu}, MmLeuRS/CP1^{Ile}, or MmLeuRS/CP1^{Val} (data not shown). This contrasts with the wild type *M. mobile* LeuRS, which is prone to mistakes that it fails to efficiently clear in the absence of the CP1 domain editing module (19).

Fusion of the CP1 domain from IleRS or ValRS to *M. mobile* LeuRS failed to cleave Ile-tRNA^{Leu}. This would be expected based on the natural editing specificities of full-length IleRS and ValRS that would block isoleucine from the amino acid-binding pocket of the hydrolytic active site (10, 29, 30). Pyrophosphate exchange assays (19, 24) support that isoleucine is activated by each of the hybrid *M. mobile* LeuRS proteins, albeit at a higher K_m as compared with the cognate leucine substrate (Fig. 3 and supplemental Table S1). However, isoleucine is not mischarged to tRNA^{Leu} by any of the hybrid LeuRS proteins that contain a CP1 domain (data not shown). In contrast, the core *M. mobile* LeuRS that is missing a CP1 domain and serves as a scaffold for these hybrid proteins produces mischarged Ile-tRNA^{Leu} (19).

Splitting the primary structure of the Rossmann fold to accommodate the addition of a CP1 domain decreased k_{cat}/K_m for leucine activation (supplemental Table S1). Remarkably, however, the fusion to either CP1^{Leu} or CP1^{Ile} dramatically enhanced discrimination of the cognate leucine substrate, relative to the noncognate aliphatic amino acids (Fig. 3 and supplemental Table S1). In the case of MmLeuRS/CP1^{Leu}, which reconstructs a canonical LeuRS from the naturally occurring *M. mobile* LeuRS, a small increase in the K_m of 3-fold for leucine contrasts to increases ranging from 24-fold for isoleucine, 35-fold for methionine, and 64-fold for valine. Similarly, the addition of CP1^{Ile} or CP1^{Val} yielded only 2–5-fold increases in K_m for leucine in the synthetic active site, as compared with at least 10-fold increases for noncognate amino acids. The addition of CP1^{Leu} and CP1^{Ile} to the canonical core of *M. mobile* LeuRS increased the discrimination factor for isoleucine 2–3-fold and lowered efficiency of isoleucine activation by almost 100-fold, based on k_{cat}/K_m ratios (Fig. 3).

Similar effects were observed for valine activation in the synthetic site of both hybrid LeuRSs. The discrimination factor for valine increased almost 9-fold, with decreasing enzymatic effi-

ciency. Although we did not observe dramatic changes in the discrimination factor for noncognate amino acids when CP1^{Val} was fused to the *M. mobile* LeuRS Rossmann fold, the efficiency of valine activation was lowered. In addition, the hybrid mutant MmLeuRS/CP1^{Val} did not display mischarging activity, similar to the CP1^{Leu} and CP1^{Ile} hybrids, and maintained similar catalytic efficiency during the aminoacylation reaction (Table 1). Thus, these mechanistic links between the fused CP1 domain and the canonical cores have diverged in an idiosyncratic way.

DISCUSSION

As protein synthesis evolved to greater sophistication, the cellular demand for fidelity of the proteome also increased. The addition of the CP1 editing domain added a hydrolytic sieve to clear aaRS aminoacylation mistakes. This double sieve model to increase aaRS fidelity has been capitalized upon by at least half of the aaRS enzyme family (15, 31).

Reports on IleRS show that its CP1 domain is intimately linked with tRNA-dependent pre-transfer editing activity (32–35). We have shown in LeuRS that removal of the CP1 domain can influence pre-transfer editing that is associated with the synthetic core (20). We hypothesize that the CP1 domain also influences adenylate stability and hydrolysis in the synthetic active site. Interestingly, the insertion editing domain (INS) of the class II bacterial prolyl-tRNA synthetase (ProRS) has been shown to play a role in facilitating or stabilizing adenylate formation (36).

Specificity and fidelity of the aaRSs are initially dependent on the finely tuned synthetic core of the enzymes, which binds and activates amino acids in an ATP-dependent mechanism for aminoacylation to tRNA. The fusion of the CP1 editing domain splits the primary structure of the ancient Rossmann fold that comprises the class I aaRS catalytic core of LeuRS, ValRS, and IleRS. Remarkably, however, rather than disrupting the enzyme core, the insertion of the LeuRS CP1 editing domain distally enhances specificity in the synthetic aminoacylation active site. Thus, fusion of these two protein domains provided a second mechanism to minimize errors that goes beyond the acquisition of the hydrolytic editing site. This finding is consistent with the previously published molecular dynamic simulation that the CP1 domain contributes to the cognate amino acid specificity in tryptophanyl-tRNA synthetase (37). It is also consistent with the editing domains of the class II ProRS, which have been fused or appended to the synthetic core at diverse sites (36, 38).

Our results support that the evolutionary fusion of the CP1 protein domain to the Rossmann fold of the aaRSs not only introduced a hydrolytic active site for editing to improve fidelity, but also enhanced substrate discrimination within the synthetic active site in the ancient catalytic core of the enzyme. This was also found for the class II ProRS editing domain, which is fused to a catalytic core that has a completely different fold (36, 38). Surprisingly, in the case of LeuRS, this increase in discrimination was also conferred by the addition of the noncognate CP1^{Ile} to LeuRS. This suggests that there is inherent overlap between CP1^{Leu} and CP1^{Ile} or in the way these domains are linked to the canonical core. Because the addition of CP1^{Val} did not significantly enhance substrate discrimination in the canonical core *per se*, it is also possible that these enzymes

acquired a common CP1 domain, which subsequently diverged to accommodate varied specificities.

Although it maintains its CP1 domain, the human mitochondrial LeuRS is similar to *M. mobile* LeuRS in that it has acquired mutations in its hydrolytic active site that have abolished its editing activity (39). Significantly, kinetic measurements showed that the synthetic site has increased its discrimination to exceed 1/3000. Likewise, yeast ProRS has an inactive editing domain that has been retained in conjunction with an active site that has similarly increased amino acid discrimination (38). This is the threshold for aaRSs that has been proposed to require an editing domain to maintain fidelity (2, 40).

It is possible that the human LeuRS enzyme retains its CP1 domain because it increases amino acid discrimination in the synthetic site similar to the hybrid *M. mobile* LeuRSs. In contrast, fidelity is decreased for the wild type *M. mobile* LeuRS due to a loss of discrimination in the aminoacylation active site, as well as the shedding of its CP1 editing domain (19). Because the *M. mobile* LeuRS has uniquely lost its CP1 domain during evolution, we hypothesize that this compromise in amino acid fidelity provides an idiosyncratic selective advantage to this host-dependent pathogen.

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