Translational Quality Control by Bacterial Threonyl-tRNA Synthetases*

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Translational fidelity mediated by aminoacyl-tRNA synthetases ensures the generation of the correct aminoacyl-tRNAs, which is critical for most species. Threonyl-tRNA synthetase (ThrRS) contains multiple domains, including an N2 editing domain. Of the ThrRS domains, N1 is the last to be assigned a function. Here, we found that ThrRSs from Mycoplasma species exhibit differences in their domain composition and editing active sites compared with the canonical ThrRSs. The Mycoplasma mobile ThrRS, the first example of a ThrRS naturally lacking the N1 domain, displays efficient post-transfer editing activity. In contrast, the Mycoplasma capricolum ThrRS, which harbors an N1 domain and a degenerate N2 domain, is editing-defective. Only editing-capable ThrRSs were able to support the growth of a yeast thrS deletion strain (ScΔthrS), thus suggesting that ScΔthrS is an excellent tool for studying the in vivo editing of introduced bacterial ThrRSs. On the basis of the presence or absence of an N1 domain, we further revealed the crucial importance of the only absolutely conserved residue within the N1 domain in regulating editing by mediating an N1–N2 domain interaction in Escherichia coli ThrRS. Our results reveal the translational quality control of various ThrRSs and the role of the N1 domain in translational fidelity.

Aminoacyl-tRNA synthetase (aaRS) catalyzes tRNA aminoacylation and generates aminoacyl-tRNA for protein biosynthesis (1–3). This process requires amino acid activation by condensation with ATP, forming the aminoacyl-adenylate, and subsequently transferring the aminoacyl moiety to the 3′ terminus of the cognate tRNA. Faithful translation of the genetic code largely relies on the correct selection and recognition of substrates by aaRSs (4). It is challenging for some aaRSs to sufficiently discriminate between cognate and noncognate amino acids because these molecules are biochemically and/or structurally similar (5). To address this difficulty, approximately half of the extant aaRSs incorporate a proofreading (editing) activity to clear misactivated aminoacyl-adenylate (pretransfer editing) and/or mischarged tRNAs (post-transfer editing) (6). Pretransfer editing is further classified into tRNA-independent or tRNA-dependent editing on the basis of the presence or absence of tRNA in stimulating editing (7, 8).

In most cases, correct aminoacyl-tRNA generation is critical for the faithful transduction of genetic information, which is supported by the high levels of amino acid conservation in editing active sites of specific aaRSs across the three domains of life. When this editing function is impaired or abolished, cellular dysfunction or disease phenotypes are often observed (9, 10). However, in a limited number of instances, the synthesis of mischarged tRNA (from either compromised editing or tRNA identity alteration) has conferred unexpected advantages. For example, translational ambiguity at the CUG Leu codons derived from the synthesis of Leu-tRNA<sub>mt</sub> by Candida albicans leucyl-tRNA synthetase (LeuRS) expands the protein primary structure on a proteome-wide scale and improves protein plasticity with morphopathogenesis (11, 12). LeuRS and phenylalanyl-tRNA synthetase (PheRS) from some Mycoplasma parasites carry point mutations or deletions in their editing domains, thus yielding mischarged tRNA<sub>Leu</sub> or tRNA<sub>Phe</sub> that is likely to escape host defense systems, because of antigen diversity from mistranslation (13–15).

Threonyl-tRNA synthetases (ThrRSs) can be divided into eukaryotic/bacterial and archaeal types (16, 17). ThrRSs belong to class II aaRS and form dimers. The domain composition of the bacterial and eukaryotic (cytoplasmic and mitochondrial) ThrRSs is well conserved. The domains include the N1 (with unknown function), N2 (for editing), aminoacylation (for amino acid activation and tRNA charging), and C-terminal (for tRNA binding) domains (CTDs) (16), along with a newly evolved N-terminal extension (N-extension) in eukaryotic enzymes (for catalysis and protein stability) (18). With the exception of Saccharomyces cerevisiae mitochondrial ThrRS (ScmtThrRS) and ThrRSs from the mitochondria of other yeasts (such as C. albicans and Schizosaccharomyces pombe) naturally lacking the editing domain (19, 20), all other known ThrRSs are capable of editing; they include Escherichia coli ThrRS (EcThrRS) (21), S. cerevisiae cytosolic ThrRS (ScThrRS)
(22), and human mitochondrial ThrRS (hmtThrRS) (23). Editing by ThrRSs takes place in the N2 editing domain (21). The reason for the editing deficiency of the ScmtThrRS is unknown but may be similar to the reasons underlying the lack of editing in other mitochondrial aaRSs, such as LeuRS (24) and PheRS (25).

The protein translation system of the *Mycoplasma* species is unique in that several aaRSs (class I LeuRS and class II PheRS) are editing-deficient, thus leading to mistranslation and the subsequent accumulation of a statistical proteome, which is likely to be responsible for antigenic diversity and phenotypic plasticity (13). In addition, on the basis of sequence analysis, ThrRS from some *Mycoplasma* species have been suggested to be editing-deficient (13). Here, we show that the ThrRS from *Mycoplasma mobile* (MmThrRS) without the N1 domain is editing-capable, whereas the ThrRS from *Mycoplasma capricolum* (McThrRS) with both the N1 and N2 domains is an error-prone aaRS, and provide a rationale for the function of *Mm*ThrRS without the N1 domain. Eukaryotic yeast cells are sensitive to editing catalyzed by introduced bacterial ThrRSs. For this reason, the yeast ThrRS knock-out strain is an excellent tool for assessing the editing of bacterial ThrRSs. Although the function of N1 has not been previously clearly assigned, our results show that the editing catalyzed by *Ec*ThrRS is mediated by the communication between the N2 editing and N1 domains.

Results

Sequence Analysis of *Mycoplasma* ThrRSs—ThrRSs from bacteria (e.g., *Ec*ThrRS) and eukaryotes (either cytoplasmic (e.g., ScThrRS) or mitochondria (e.g., hmtThrRS)) contain well conserved N1, N2, aminoacylation, and CTDs; the eukaryotic enzymes have an additional N-extension domain. The ScmtThrRS is an exception because it consists of only the aminoacylation and CTDs (19). Detailed primary sequence analysis of ThrRSs from *Mycoplasma* species has revealed the diversity of their domains. Despite having a domain composition similar to that of *Ec*ThrRS, the active site of the McThrRS N2 editing domain was degenerate (Fig. 1A). Counterparts of crucial residues with functional side chains in the editing active site of *Ec*ThrRS (including His73, His77, Lys156, Asp180, Cys182, and His186) (bold and light green in Fig. 1B) (21) have been extensively mutated to Leu69, Phe73, Gln150, Met174, Val176, and Leu180 (bold and red in Fig. 1B), thus raising the question of how this enzyme maintains translational quality control. *Mm*ThrRS lacks an N1 domain (with unknown evolutionary advantages) but maintains the intact editing active site of the N2 domain (bold and yellow in Fig. 1B). Excluding ThrRSs containing only the aminoacylation and CTDs (such as yeast mitochondrial ThrRSs), to our knowledge, the *Mm*ThrRS is the first example of a ThrRS naturally lacking an N1 domain. Similarly to ScmtThrRS, the *Mycoplasma wenyonii* ThrRS (MwThrRS) has retained only the aminoacylation and CTDs. Although the ThrRS from *Mycoplasma hemocanis* (MhThrRS) has only aminoacylation and CTDs, its N terminus possesses an additional domain with no significant homolog and for which no function has been assigned. Thus, the ThrRSs from *Mycoplasma* species exhibit the most distinct diversity in the composition and arrangement of their domains (Fig. 1A).

*Mm*ThrRS is Editing-capable, Whereas *Mc*ThrRS is an Error-prone aaRS—The features of ThrRSs harboring only the aminoacylation and CTDs have been studied using ScmtThrRS as a model (19, 20). Thus, in this work, we focused on the study of *Mc*ThrRS (with a degenerate editing domain) and *Mm*ThrRS (with no N1 domain). Because these two enzymes are modified near or at the editing domain, we initially intended to study whether they have editing capability. The editing requirement is based on whether an aaRS is able to discriminate between cognate and noncognate amino acids with a sufficient discrimination factor (DF) (≤1/3300) (26). The DF can be calculated from kinetic constants using the equation DF = (kcat/Km)cognate amino acid/(kcat/Km)noncognate amino acid. We purified *Mm*ThrRS and *Mc*ThrRS to homogeneity (Fig. 1C). The kinetics of *Mm*ThrRS and *Mc*ThrRS for cognate Thr and noncognate Ser was determined with an ATP-PPi exchange reaction. We found that the two enzymes displayed DFs of ~1/900 (1/944 and 1/848 for *Mc*ThrRS and *Mm*ThrRS, respectively) (Table 1), which are significantly larger than the acceptable threshold (1/3300) (26). Thus, we concluded that each of these enzymes misacylates noncognate Ser and therefore is likely to require an editing function to ensure the correct tRNAThr formation.

To directly monitor the editing activities of the two enzymes, we prepared Ser-tRNAThr by using the editing-deficient *Ec*ThrRS-H73A/H77A (21). We found that *Mm*ThrRS, but not *Mc*ThrRS, was able to hydrolyze Ser-tRNAThr and remove noncognate Ser (Fig. 2A), thus indicating that the degeneration of the crucial editing active sites of *Mc*ThrRS impairs its post-transfer editing. In contrast, *Mm*ThrRS, which has an intact N2 domain, has post-transfer editing activity. We further mutated two conserved His residues of the *Mm*ThrRS N2 domain (His9 and His153), whose counterparts participate in the editing activities of *Ec*ThrRS (His73 and His77) (21) and ScThrRS (His151 and His155) (22). The *Mm*ThrRS-H9A/H13A mutant had aminoacylation activity (data not shown) similar to that of *Mm*ThrRS, but its post-transfer editing was not detected in vitro, thus indicating that the N2 domain is involved in post-transfer editing (Fig. 2A).

Furthermore, the misaminoacylation of tRNAThr with Ser by *Mc*ThrRS, *Mm*ThrRS, and *Ec*ThrRS (as an editing-capable control) was performed. We found that Ser-tRNAThr was apparently formed by *Mc*ThrRS, whereas only trace amounts of Ser-tRNAThr were formed by *Mm*ThrRS, which was comparable with *Ec*ThrRS (Fig. 2, B and C).

These data indicate that *Mm*ThrRS is an editing-capable ThrRS, whereas the *Mc*ThrRS has lost its post-transfer editing activity, probably because of its degenerate editing active site in the N2 domain. Thus, because it clearly synthesizes mischarged tRNAThr, *Mc*ThrRS is an error-prone tRNA synthetase.

*Mc*ThrRS and *Mm*ThrRS Have Negligible tRNA-dependent Pretransfer Editing Capacities—In addition to their post-transfer editing capacity, eukaryotic cytoplasmic ThrRSs (e.g., ScThrRS) also exhibit tRNA-independent and tRNA-dependent pretransfer editing activities, contributing the highest pro-
A Ser-included AMP formation assay was used to clarify whether McThrRS and MmThrRS also possess pretransfer editing activities. According to this approach, the $k_{\text{obs}}$ values of AMP formation for MmThrRS were $0.093 \pm 0.011$ and $0.086 \pm 0.010$ s$^{-1}$ in the absence and presence of tRNAThr, respectively (Fig. 3, A and C), whereas the $k_{\text{obs}}$ values of AMP formation for McThrRS were $0.224 \pm 0.027$ and $0.225 \pm 0.032$ s$^{-1}$, respectively, which were larger than those for MmThrRS (Fig. 3, B and D, and Table 2).

We also performed Thr-included AMP formation assays in the absence of tRNAThr with MmThrRS and McThrRS. The result showed that $k_{\text{obs}}$ value of MmThrRS for AMP formation was too low to be accurately determined (Fig. 4A), whereas that of McThrRS was $0.013 \pm 0.004$ s$^{-1}$ with Thr (Fig. 4, B and C), significantly lower than that with Ser ($0.224 \pm 0.027$ s$^{-1}$), indi-
cating that the observed AMP formation in the absence of tRNAThr was derived from tRNA-independent pretransfer editing but not misactivation of Ser and then the subsequent hydrolysis of Ser-AMP. Ser-AMP formation by McThrRS was more apparent than that of MmThrRS in the absence (0.020 ± 0.005 and 0.068 ± 0.018 s⁻¹ for MmThrRS and McThrRS, respectively) or presence (0.008 ± 0.002 and 0.072 ± 0.028 s⁻¹ for MmThrRS and McThrRS, respectively) of tRNA (Fig. 3, A and B, and Table 2). Furthermore, the kobs value of AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of Mm ThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS. The kobs value of Ser-AMP formation of post-transfer editing-defective MmThrRS-H9A/H13A was 0.091 ± 0.017 s⁻¹ in the presence of tRNAThr, which was comparable with that of MmThrRS.
AMP formation of MmThrRS-H9A/H13A was 0.021 ± 0.007 s\(^{-1}\) in the presence of tRNA\(^{\text{Thr}}\), which was larger than that of MmThrRS (Table 2). These data clearly show that tRNA\(^{\text{Thr}}\) does not stimulate tRNA-dependent pretransfer editing of MmThrRS and McThrRS.

McThrRS Is Unable to Complement the Loss of Yeast thrS in Vivo, Because of Its Lack of Editing Activity—We have previously constructed a ThrRS-deficient S. cerevisiae strain (Sc\(^{-}\)thrS) as an in vivo tool to assess the functions of various ThrRSs (22). To determine whether MmThrRS and McThrRS could complement the Sc\(^{-}\)thrS and to evaluate the effects of defective editing of ThrRS on yeast phenotypes, we introduced the McThrRS and MmThrRS genes into Sc\(^{-}\)thrS and observed the growth of the transformants on selective medium. Interestingly, only MmThrRS was able to rescue the Sc\(^{-}\)thrS phenotypes in the presence of 5-fluoroorotic acid (5-FOA) (Fig. 5A). The inability of the McThrRS to rescue Sc\(^{-}\)thrS growth might have originated from its failure to aminoacylate yeast tRNA\(^{\text{Thr}}\) isoacceptors or to perform post-transfer editing. To distinguish between these two possibilities, we assayed the aminoacylation of the three yeast isoacceptors tRNA\(^{\text{Thr}}\) (UGU), tRNA\(^{\text{Thr}}\) (CGU), and tRNA\(^{\text{Thr}}\) (AGU) by McThrRS. Our data showed that McThrRS aminoacylated each yeast tRNA\(^{\text{Thr}}\) isoacceptor with a higher catalytic rate than did MmThrRS (Fig. 5B), thus indicating that the defective editing function of McThrRS prevents the yeast growth.

To determine whether editing produces phenotype differences, we replaced the N1 and N2 domains of McThrRS (Met\(^{1}\)–Gly\(^{214}\)) with the N2 domain of MmThrRS (Met\(^{1}\)–Gly\(^{157}\)) to obtain the chimeric McThrRS-MmN2–ΔN1 (without N1) (Fig. 5C). McThrRS-MmN2–ΔN1 had an aminoacylation activity comparable with that of McThrRS (data not shown). An in vitro hydrolytic assay of Ser-tRNA\(^{\text{Thr}}\) showed that the transduction of the N2 domain of MmThrRS restored the post-transfer editing capability of McThrRS (Fig. 5D). Furthermore, the chimeric enzyme in Sc\(^{-}\)thrS was able to rescue cell growth, further suggesting that the defective editing of McThrRS blocks yeast growth in vivo (Fig. 5E).

To further reveal whether the editing-defective ThrRS leads to a growth failure in yeast, we introduced MmThrRS-H9A/H13A into Sc\(^{-}\)thrS. Mutations at these two editing-cruicial residues resulted in slow growth, thus clearly indicating that impaired post-transfer editing impeded normal yeast growth (Fig. 5A). It is possible that although the in vitro assay suggested that the post-transfer editing of MmThrRS-H9A/H13A (Fig. 2A) was reduced compared with that of MmThrRS, the in vivo mutation of more crucial residues was required to abolish the post-transfer editing. Thus, in the context of MmThrRS-H9A/H13A, we additionally mutated all of the editing-cruical residues (including Lys\(^{86}\), Asp\(^{117}\), Cys\(^{119}\), and His\(^{123}\)) (shown in bold and yellow in Fig. 1B) to obtain MmThrRS-N2M (Fig. 5C). The post-transfer editing of MmThrRS-N2M was abolished by these mutations (Fig. 5D), and Sc\(^{-}\)thrS harboring MmThrRS-N2M did not grow (Fig. 5F). However, in the absence of 5-FOA, by Western blot analysis, we detected no differences in either the in vivo amounts of MmThrRS, MmThrRS-H9A/H13A, and MmThrRS-N2M in Sc\(^{-}\)thrS on the basis of a His\(^{6}\) tag at the C terminus of each protein (Fig. 5G) or the in vitro aminoacylation of tRNA\(^{\text{Thr}}\) by these three enzymes (data not shown). Thus, the loss of editing capacity in these mutants is responsible for their failure to rescue the growth defects of Sc\(^{-}\)thrS.

These observations suggest a correlation between the growth phenotype of Sc\(^{-}\)thrS and the editing capacities of various native or mutant ThrRSs (Fig. 5C). The findings also clearly show that Sc\(^{-}\)thrS cells are highly sensitive to post-transfer editing catalyzed by introduced bacterial ThrRSs. Therefore, Sc\(^{-}\)thrS is an ideal tool to study not only aminoacylation activity (22) but also the post-transfer editing activity of exogenous bacterial ThrRSs in vivo in subsequent studies.

Absence of the N1 Domain of MmThrRS Increases in Vivo Activity and Optimizes Protein Structure/Stability—To better understand the consequences of the absence of the N1 domain from MmThrRS, we fused the N1 domain of McThrRS (Met\(^{1}\)–Leu\(^{67}\)) at the N terminus of MmThrRS, resulting in the chimeric mutant MmThrRS–\(^{-}\)McN1 (Fig. 5C). MmThrRS–\(^{-}\)McN1 was unable to rescue cell growth in the presence of 5-FOA (Figs. 5C and 6A), indicating that the activity and/or protein level of the chimeric mutant had been significantly decreased. Western blot analysis of the yeast cell lysate showed that under the same conditions, the amount of MmThrRS–\(^{-}\)McN1 dropped sharply to ~20% of that of MmThrRS, indicating that maintenance of the N1 domain of McThrRS has a negative effect on MmThrRS protein structure and/or stability. However, the MmThrRS–\(^{-}\)McN1 expression level was comparable with that of Sc\(^{-}\)thrS (Fig. 6, B and C), implying that the protein activity was also influenced by the presence of the N1 domain of McThrRS. We
**FIGURE 5. Editing-defective ThrRSs are unable to rescue ScThrRS loss of function yeast.** A, complementation of an ScThrRS loss of function mutant strain by MmThrRS, MmThrRS-H9A/H13A, and McThrRS. ScThrRS and p425TEF are used as positive and negative controls, respectively. B, aminoacylation of yeast tRNA\textsuperscript{Thr} isoacceptors by MmThrRS or McThrRS. C, schematic showing the domain compositions of N1 and N2 for the EcThrRS, MmThrRS, McThrRS enzymes, and the various mutants with domain deletions, swaps, or mutations. The aminoacylation and CTDs are shown as the “main body” for clarity. The capacity of the proteins to support the growth of ScThrRS (complementation) or post-transfer editing is indicated with + and − symbols. ND represents not determined because we were unable to obtain soluble proteins. Two asterisks in the N2 domain indicate degeneracy (in McThrRS) or mutation (in MmThrRS) of the active sites. D, post-transfer editing of the Ser-tRNA\textsuperscript{Thr} by the McThrRS-MmmN2-A\textsuperscript{N1} ( ) and McThrRS-N2M ( ). E, rescue of the ScThrRS loss-of-function strain by McThrRS, McThrRS-MmN2 and McThrRS-MmmN2-A\textsuperscript{N1}. EcThrRS and p425TEF are shown as positive and negative controls, respectively. F, rescue of the ScThrRS loss of function strain by MmThrRS-N2M. MmThrRS and p425TEF are shown as positive and negative controls, respectively. G, steady-state protein levels of MmThrRS, MmThrRS-H9A/H13A, and MmThrRS-N2M, each of which had a His\textsubscript{6} tag at the C terminus. ScThrRS expressed from a rescue plasmid had no His\textsubscript{6} tag at the C terminus. GAPDH was used as the loading control. The data shown in B and D represent averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.
were unable to purify \( Mm \text{ThrRS} \cdot \cdot \cdot \cdot \text{McN1} \) from the \( E. coli \) transformants because of the formation of inclusion bodies. Similarly, after the replacement of the \( \text{McThrRS} \cdot \cdot \cdot \cdot \text{N2} \) domain (Asn\(^{68} \)-Gly\(^{214} \)) with its \( Mm \text{ThrRS} \) equivalent (Asn\(^{8} \)-Gly\(^{157} \)), the chimeric \( Mm \text{ThrRS} \cdot \cdot \cdot \cdot \text{MnN2} \) formed inclusion bodies in the \( E. coli \) transformants (data not shown) and was unable to rescue \( Sc \Delta \text{thrS} \) growth (Figs. 5C and 6D). However, \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{MnN2} \cdot \cdot \cdot \cdot \text{AN1} \) was functional both in vitro and in vivo (Fig. 5, D and E). The phenotypic differences between the \( Mm \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{McN1} \) or \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{MnN2} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{MnN2} \cdot \cdot \cdot \cdot \text{AN1} \) (only differing in the absence or presence of the \( N1 \) domain of \( Mc \text{ThrRS} \) in each pair) (Fig. 5C) imply that the functional \( N2 \) domain of \( Mm \text{ThrRS} \) is incompatible with the \( N1 \) domain of \( Mc \text{ThrRS} \). Together, the above data suggest that the absence of an \( N1 \) domain in \( Mm \text{ThrRS} \) increases its catalytic efficiency and stability.

The \( N1 \) Domain Is Essential for Editing by \( Ec \text{ThrRS} \)—By aligning ThrRSs with post-transfer editing activity from various species, several mutations were identified in the editing active site in the \( N2 \) domain of \( Mc \text{ThrRS} \) (Fig. 1A). Although functions have been assigned to the other \( T \)hRS domains, the function of the \( N1 \) domain has remained unknown. To investigate the role of the \( N1 \) domain in the context of the full-length ThrRS, we deleted the \( N1 \) domain (Met\(^{1} \)-Asn\(^{58} \)) of \( Mc \text{ThrRS} \) to obtain the deletion mutant \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) (Lys\(^{59} \)-Lys\(^{639} \)) (Fig. 5C). As expected, \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) did not rescue the growth of \( Sc \Delta \text{thrS} \) (Figs. 5C and 6D). Recombinant \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) was successfully purified to high homogeneity from \( E. coli \) transformants (Fig. 1C), and its kinetic constants in the aminocytlation reaction for three substrates were measured. As compared with those of \( Mc \text{ThrRS} \), the \( K_{m} \) values of \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) for Thr and ATP were little changed, whereas the \( K_{m} \) values for tRNA\(^{\text{Thr}} \) was decreased (2.00 ± 0.28 of \( Mc \text{ThrRS} \) versus 0.94 ± 0.17 \( \mu \)M of \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \)). The \( k_{cat} \) values for the three substrates were marginally decreased after truncation. Overall, there was little change in the enzymatic activities after the deletion of the \( Mc \text{ThrRS} \) \( N1 \) domain (Table 3). As anticipated, \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) was deficient in post-transfer editing similar with native \( Mc \text{ThrRS} \) (data not shown).

Because the editing deficiency of \( Mc \text{ThrRS} \) and its inability to support the growth of \( Sc \Delta \text{thrS} \), we were unable to assess the contribution of the \( N1 \) domain to the editing activity of ThrRS and its in vivo function. Therefore, we further used \( Ec \text{ThrRS} \), which has editing function (21) and was able to replace yeast ThrRS for in vivo aminocytlation (Fig. 6A). An \( Ec \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) (Lys\(^{63} \)-Glu\(^{642} \)) expression vector was constructed, and the enzyme was purified (Fig. 1C). Consistently with the in vitro data of aminocytlation kinetics from \( Mc \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) (Table 4), \( N1 \) truncation did not have an observable influence, thus further showing that the \( N1 \) domain contributes little to substrate binding and tRNA charging. To determine whether this \( N1 \) truncation influences the enzyme structure, we determined the dissociation constant \( (k_{d}) \) values of the native \( Ec \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) with tRNA\(^{\text{Thr}} \) by using a filter-binding assay. The \( k_{d} \) values for \( Mc \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) were 1.92 ± 0.23 and 1.54 ± 0.20 \( \mu \)M, respectively, indicating that the \( N1 \) truncation has a negligible effect on the protein structure (Fig. 7A). However, the \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) enzyme did not support the growth of \( Sc \Delta \text{thrS} \) (Figs. 5C and 7B). Thus, we speculated that editing is impaired in \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \). Indeed, the hydrolysis of Ser-tRNA\(^{\text{Thr}} \) revealed that \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) had no post-transfer editing (Fig. 7C). The editing deficiency and failure to rescue \( Sc \Delta \text{thrS} \) containing \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) were reminiscent of the characteristics of \( Mc \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{N2M} \), thus strongly suggesting that \( Sc \Delta \text{thrS} \) cell viability is directly regulated by the ThrRS editing function. Both \( Mc \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) lack tRNA-dependent pretransfer editing capacity. To elucidate whether \( Ec \text{ThrRS} \) has tRNA-dependent and tRNA-dependent pretransfer editing activities, along with any potential role of the \( N1 \) domain, we assayed AMP formation catalyzed by \( Ec \text{ThrRS} \) and \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \), respectively. The amount of AMP produced by \( Ec \text{ThrRS} \) through a TLC-based editing assay was significantly stimulated in the presence of tRNA (Fig. 7, D and E), and the calculated \( k_{obs} \) values were 1.81 ± 0.21 s\(^{-1} \) with tRNA and 0.33 ± 0.05 s\(^{-1} \) without tRNA, thus indicating strong tRNA-dependent editing, including the pre- and post-transfer editing of \( Ec \text{ThrRS} \) (Table 5). However, under the same conditions, the \( k_{obs} \) values of the \( Mc \text{ThrRS} \cdot \cdot \cdot \cdot \text{AN1} \) in the presence and absence of tRNA\(^{\text{Thr}} \) were only slightly different (0.34 ± 0.06 s\(^{-1} \) with tRNA and 0.25 ±
Proofreading by Various Threonyl-tRNA Synthetases

The Importance of the N1 Domain for Editing EcThrRS Relies on the Interdomain Communication—Given the importance of the N1 domain in editing of EcThrRS and in supporting the growth of ScΔthrS, we subsequently studied its mechanism.

The editing active site of the ThrRS is within the N2 domain; however, our above data showed that the N1 domain does not influence tRNA-independent pretransfer editing, which is considered to occur at the synthetic active site (19, 27), and further implying that the N1 domain truncation does not affect the protein structure.

The data show that tRNA triggers little AMP formation by EcThrRS-ΔN1 without post-transfer editing and that EcThrRS has negligible tRNA-dependent pretransfer editing activity, similarly to MmThrS and McThrS (Fig. 3). In addition, EcThrRS and EcThrRS-ΔN1 have similar tRNA-independent pretransfer editing activity levels, thus suggesting that the N1 domain does not influence tRNA-dependent pretransfer editing, which is considered to occur at the synthetic active site (19, 27), and further implying that the N1 domain truncation does not affect the protein structure.

The Impact of the ΔN1 Domain on EcThrRS-The N1 domain is located at the N terminus of the N2 editing domain, far away from the charged tRNA Thr synthesized in the aminoacylation active site (28). On the basis of its spatial localization, we propose that interdomain communication is likely between the N1 and N2 domains. We analyzed the primary and tertiary structures of the available ThrRS crystal structures. On the basis of the tertiary structure of EcThrRS (Protein Data Bank code 1QF6) (16), the Asp46 residue in the N1 domain might potentially play a role in the N1-N2 interaction because: (i) the Asp residue is the only absolutely conserved residue within the N1 domain in all ThrRSs harboring the N1 domain (Fig. 8A), and (ii) Asp46 is located at the interface between the N1 and N2 domains, and its side chain points toward the N2 domain (Fig. 8B). To uncover the role of Asp46 in the in vivo function of EcThrRS, Asp46 was mutated to nine other residues (Ala, Arg, Glu, Tyr, Asn, Cys, Leu, Pro, and Thr). Each gene encoding these variants was then expressed in ScΔthrS. Strikingly, none of the Asp46 mutants were able to rescue the growth of the thrS deletion strain (Fig. 8C), including the Glu46 substitution variant with the similarly negative charge (EcThrRS-D46E). The results indicated that both the charge and size of the side chain at residue 46 are crucial to the growth of ScΔthrS. To determine whether the growth deficiency was due to the loss of editing and/or aminoacylation activity, we selected EcThrRS-D46R with a positively charged residue at position 46 and EcThrRS-D46E with negatively charged residue at this position as the representative samples and then investigated their in vitro aminoacylation and editing activities relative to the EcThrRS. Although EcThrRS-D46R and EcThrRS-D46E had similar aminoacylation activities as the mutants with EcThrRS (Fig. 8D), their post-transfer editing activity was completely lost (Fig. 8E), thus suggesting that a loss of editing activity was responsible for the failure to rescue. Because Asp46 is located in the N1 domain, it is likely that Asp46 mediates communication between the two domains by its interaction with some residues in the N2 domain to regulate the editing activity of EcThrRS.

By carefully analyzing the structure of EcThrRS (Protein Data Bank code 1QF6) (16), we found that an oxygen atom of the carboxyl group side chain of Asp46 potentially interacts with the side chain of Lys136 (2.95 Å) or Tyr173 (2.66 Å) in the N2 domain, whereas another oxygen atom of Asp46 likely interacts with the amide group of the His186 main chain (2.83 Å) in the N2 domain (Figs. 8B and 9A). The Lys136 mutants substituted with Ala, Glu, and Arg were tested for their capacity to rescue the growth of the ScΔthrS strain. EcThrRS-K136E was unable to replace ScThrS; however, both EcThrRS-K136A and EcThrRS-K136R compensated for the loss of ScThrS and EcThrRS (Fig. 9B). These data indicated that the interaction of Asp46 with other potential residues was sufficient for in vivo function in EcThrRS-K136A and EcThrRS-K136R. Indeed, introduction of a Glu residue at position 136 leads to carboxylate groups of both Glu136 and Asp46 into close proximity, inducing structural disruption, thus accounting for the failure of EcThrRS-K136E to rescue growth of ScΔthrS. In contrast, Ala136 in EcThrRS-K136A and Arg136 in EcThrRS-K136R are accommodated both in terms of size (a small methyl group) or complementary charge. After substitution of Tyr173 with Ala, Asp, or Arg, none of the three mutants, EcThrRS-Y173A, -Y173R, or -Y173D, rescued the growth of ScΔthrS, thus implying that Tyr173 is crucial for the in vivo function of EcThrRS (Fig. 9B). The purified EcThrRS-Y173R and EcThrRS-Y173D had modest reductions in their aminoacylation activity compared with EcThrRS (within 2-fold) (Fig. 9C); however, their post-transfer editing activity was abolished (Fig. 9D), again indicating that mutants unable to perform post-transfer editing could not complement the loss of thrS in ScΔthrS, similarly to EcThrRS-ΔN1, EcThrRS-D46R, and EcThrRS-D46E.

The above data show the importance of the Asp46 in the N1 domain and the Tyr173 in the N2 domain. If these two residues directly interact, such an interaction could be re-established by the simultaneous mutation of the two residues. We constructed five double mutants in the context of EcThrRS-D46E, including EcThrRS-D46E/Y173H, -D46E/Y173K, -D46E/Y173S, EcThrRS-D46E/Y173F, and -D46E/Y173R. Only EcThrRS-D46E/Y173F was able to support ScΔthrS growth, despite its slightly decreased efficiency, thus suggesting a re-established interaction between the N1 and N2 domains (Fig. 9E). EcThrRS-D46E did not rescue ScΔthrS growth, and EcThrRS-Y173F provided poor support for growth compared with EcThrRS, thus suggesting that simultaneous mutations at both the 46 and 173 positions are required for the recovery of interaction between the N1-N2 domains (Fig. 9E). As described above, Asp46 potentially also interacts with the main chain amide group of His186. When His186 was substituted with Gly (EcThrRS-H186G) to minimize the side chain and thereby enhance the flexibility, the EcThrRS-H186G complemented ScThrRS very well.

### Table 4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{cat}$/M·s$^{-1}$</th>
<th>$k_{cat}/K_{m}$</th>
<th>$k_{cat}/K_{m}$</th>
</tr>
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<tbody>
<tr>
<td>EcThrRS</td>
<td>Thr</td>
<td>0.69 ± 0.08</td>
<td>108.33 ± 11.24</td>
<td>6.37</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>0.63 ± 0.09</td>
<td>36.71 ± 2.97</td>
<td>17.16</td>
</tr>
<tr>
<td>EcThrRS-ΔN1</td>
<td>Thr</td>
<td>0.64 ± 0.06</td>
<td>168.02 ± 18.61</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>0.63 ± 0.05</td>
<td>35.17 ± 4.18</td>
<td>19.71</td>
</tr>
</tbody>
</table>

0.04 s$^{-1}$ without tRNA (Fig. 7, F and G, and Table 5). These data show that tRNA triggers little AMP formation by EcThrRS-ΔN1 without post-transfer editing and that EcThrRS has negligible tRNA-dependent pretransfer editing activity, similarly to MmThrS and McThrS (Fig. 3). In addition, EcThrRS and EcThrRS-ΔN1 have similar tRNA-independent pretransfer editing activity levels, thus suggesting that the N1 domain does not influence tRNA-dependent pretransfer editing, which is considered to occur at the synthetic active site (19, 27), and further implying that the N1 domain truncation does not affect the protein structure.
Proofreading by Various Threonyl-tRNA Synthetases

FIGURE 7. Role of N1 domain of EcThrRS. A, $k_d$ determinations for EcThrRS (●) and EcThrRS-ΔN1 (■) for tRNA$^\text{Thr}$, yeast complementation by EcThrRS and EcThrRS-ΔN1. ScThrRS and p425TEF were used as positive and negative controls, respectively. C, post-transfer editing of Ser-tRNA$^\text{Thr}$ by EcThrRS (●) and EcThrRS-ΔN1 (■). The spontaneous hydrolysis of Ser-tRNA$^\text{Thr}$ (●) is included as a negative control. D–G, generation of [32P]AMP in the absence (+tRNA) or presence (−tRNA) of tRNA$^\text{Thr}$ by EcThrRS (D and E) and EcThrRS-ΔN1 (F and G), after incubations of 2, 4, 6, 8, and 10 min. A 2-fold dilution of (α-32P)ATP (initial concentration, 3 μM) was included for quantification. F and G, quantification of AMP formation by EcThrRS (F) and EcThrRS-ΔN1 (G) with or without tRNA. The data in A, C, E, and G represent averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.

TABLE 5 $k_{\text{obs}}$ values of EcThrRS and EcThrRS-ΔN1 in AMP formation assays

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>tRNA</th>
<th>$k_{\text{obs}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcThrRS</td>
<td>−</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.81 ± 0.21</td>
</tr>
<tr>
<td>EcThrRS-ΔN1</td>
<td>−</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.34 ± 0.06</td>
</tr>
</tbody>
</table>

(data not shown). Interestingly, when Gly$^{186}$ was introduced into the complementation-defective EcThrRS-D46E, the growth of the yeast containing EcThrRS-D46E/H186G was restored to levels comparable with those of the native enzyme (Fig. 9E), thus implying that the bulkier Glu at position 46 clashes with the main chain of His$^{186}$. However, the introduction of the smallest Gly at 186 improved the communication.

The aminoacylation and post-transfer editing activities of the purified EcThrRS-D46E/Y173F and EcThrRS-D46E/H186G were assayed. Their aminoacylation activity was decreased only slightly (Fig. 9C), whereas their post-transfer editing activity was modestly restored (Fig. 9F). This slight increase in the editing seemed sufficient to support in vivo translational quality control. Collectively, these data demonstrate that there is an interdomain communication mechanism mediated by an interaction between residues Asp$^{46}$ in the N1 domain and Lys$^{136}$, Tyr$^{173}$, and His$^{186}$ in the N2 domain, which is critical for the editing active site in the N2 domain and cell survival.

Discussion

Eukaryotic Cells Are Reliant on ThrRS Editing Activity—In the present work, we revealed that the growth of yeast cells depends on the editing function of ThrRS, as illustrated by complementation with MmThrRS, McThrRS-MmN2-ΔN1, and EcThrRS or non-complementation with McThrRS and EcThrRS-ΔN1. ScΔthrS containing MmThrRS-H9A/H13A grew poorly compared with ScΔthrS containing MmThrRS, whereas MmThrRS-N2M did not support the growth of ScΔthrS. Notably, editing activity was not detected in vitro for either MmThrRS-H9A/H13A or McThrRS-N2M. It is possible that MmThrRS-H9A/H13A retains trace editing activity in vivo that is sufficient for basal translational quality control, whereas MmThrRS-N2M has no in vivo editing activity. Indeed, only trace editing activity is sufficient for supporting the normal growth of yeast cells, illustrated by EcThrRS-D46E/Y173F and EcThrRS-D46E/H186G. EcThrRS-Y173F supported yeast growth very poorly, suggesting that the hydrogen bond between Asp$^{46}$ and Tyr$^{173}$ is important. However, EcThrRS-D46E/Y173F was functional in supporting yeast growth; it should have no hydrogen bond between Glu$^{46}$ and Phe$^{173}$ in EcThrRS-D46E/Y173F. In addition, based on structure of
EcThrRS (Protein Data Bank code 1QF6) (16), it is clear that the aromatic ring of Tyr173 stacks on the imidazole group of His186 on one face while packing against the aliphatic portion of the Lys136 side chain on the other face. Thus, we suggest that Tyr173 maintains a hydrophobic core and that the salt bridge between Asp46 to Lys136 is not sufficient to sustain the fold when a cavity introduced by only mutation at Tyr173.

EcThrRS-D46E/H186G supports growth of ScH9004 thrS well, indicating that the vacant position created by Gly at position 186 is compensated by nearby residues, possibly His73 or His77. Altogether, these results suggest that the network is complex and requires multiple interactions. Indeed, mutations at both Asp46 and Tyr173 can restore yeast growth. The assay of the editing activity of bacterial ThrRSs with ScH9004 thrS was more sensitive than with the in vitro hydrolysis of Ser-tRNA^Thr. Hence, ScΔthrS is an excellent tool for studying the in vivo editing function of introduced bacterial ThrRSs.

Hidden Contribution of N1 Domain to the Editing Function of ThrRS—Using ScThrRS, we have previously shown that the N-extension of eukaryotic ThrRS is involved in the enzymatic activity, structure, and stability of ThrRS (18). However, the biological significance of the N1 domain of ThrRS has so far remained elusive. EcThrRS has N1, N2, aminoacylation, and CTDs. By aligning sequences from various species, we determined that MmThrRS did not have an N1 domain. By constructing EcThrRS-ΔN1, we found that the N1 domain is directly involved in the editing function of ThrRS. Indeed, the effect of the N1 domain on the editing function of ThrRS had been ignored until now because of its spatial separation from the editing active site of the N2 domain. Asp46, the only absolutely conserved amino acid residue in the N1 domain of EcThrRS, forms an interaction network with the Lys136, Tyr173, and His186 in the N2 domain. Substitution of Asp46 with any other amino acid, even Glu or Asn, resulted in a loss of the editing activity of EcThrRS, thus suggesting that both the property and the size of this residue play critical roles in this interdomain communication. We propose that an interaction network among Asp46, Lys136, Tyr173, and His186 controls the spatial orientation or rotation of the N2 editing domain during the translocation of the CCA^76 end from the aminoacylation active site to the editing active site. Disruption of this interaction by a mutation at Asp46 or Tyr173 inhibited the CCA^76 end with Ser from being captured or accommodated by the N2 domain. Indeed, the rotation of the CP1 editing domain, a prerequisite for a proper accommodation of the CCA^76 end of mischarged tRNAs, has been observed in class I aaRSs, LeuRS, and valyl-tRNA synthetase (29–31). However, the absence of the N1 domain was beneficial for the function and structure of

FIGURE 8. The Asp^46 of the N1 domain controls editing and cell viability. A, sequence alignment of the N1 domains of ThrRSs from various species. Ec, E. coli; Mc, M. capricolum; Sa, S. aureus; Aa, A. aeolicus; Tf, T. thermophiles; Sc, S. cerevisiae; Hs, Homo sapiens. B, crystal structure of the EcThrRS–tRNA^Thr complex (Protein Data Bank code 1QF6) (16) showing the spatial location of the Asp^46-containing N1 domain and the Tyr^173- and His^186-containing N2 domain. Protein domains are labeled and shown in different colors, with tRNA in orange. C, yeast complementation results for the various Asp^46 mutants. D, aminoacylation of tRNA^Thr by the native EcThrRS (●), EcThrRS-D46R (□), and EcThrRS-D46E (○). E, post-transfer editing of Ser-tRNA^Thr by EcThrRS (●), EcThrRS-D46R (□), and EcThrRS-D46E (○). The spontaneous hydrolysis of Ser-tRNA^Thr (△) was included as a negative control. The data in D and E represent averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.
MmThrRS in vivo. Even without an N1 domain, the MmThrRS retained post-transfer editing activity. It is possible that the N2 domain of MmThrRS is more flexible than that of EcThrRS and that this flexibility is responsible for the translocation of the CCA₇₆ end of tRNA during editing.

Mycoplasma Species May Differ in Requirement of Editing—LeuRSs from three Mycoplasma species (M. mobile, M. agalactiae, and M. synoviae) exhibit either loss or partial truncation of the editing domain, thus leading to editing deficiency and Ile misincorporation of the Leu codons in vivo (13). Similarly, the editing active sites of PheRS from M. mobile (MmPheRS) are degenerate, thus leading to Tyr replacement at the Phe codons in vivo (15). Mistranslation in the proteome of M. mobile has been suggested to be an evolutionary strategy to confer phenotypic plasticity in Mycoplasma pathogens. However, it is notable that some Mycoplasma species have retained editing-functional LeuRSs or PheRSs (13, 15). Considering ThrRS, McThrRS is degenerate in the N2 domain, similarly to the MmPheRS and LeuRS from M. synoviae. However, MmThrRS harbors an intact editing domain with post-transfer editing activity. MwThrRS consists of only aminoacylation and CTDs and thus is predicted to be editing-deficient. Interestingly, MmThrRS is a ThrRS without an N1 domain. The presence of post-transfer editing in MmThrRS and the absence of post-transfer editing in McThrRS probably reflect the different levels of mistranslation and the requirements of translational quality.

**FIGURE 9. Recovery of editing activity and yeast complementation.** A, spatial localization of Asp⁴⁶, Lys¹³⁶, Tyr¹⁷³, and His¹⁸⁶. The side chains of the Asp⁴⁶, Lys¹³⁶, and Tyr¹⁷³ residues and the main chain of the His¹⁸⁶ residue are shown as sticks (left) and spheres (right). The distance between Asp⁴⁶ and the other residues is indicated. B, yeast complementation by mutants of the Lys¹³⁶ and Tyr¹⁷³ residues. C, the aminoacylation activity of the EcThrRS (●), EcThrRS-Y173R (□), EcThrRS-Y173D (△), EcThrRS-D46E/Y173F (▲), and EcThrRS-D46E/H186G (■) variants. D, post-transfer editing of Ser-tRNAThr by EcThrRS-Y173R (□) and EcThrRS-Y173D (△). Spontaneous hydrolysis (●) and post-transfer editing of Ser-tRNAThr by EcThrRS (■) are included as negative and positive controls, respectively. E, yeast complementation by EcThrRS-D46E, EcThrRS-Y173F, EcThrRS-D46E/Y173F, and EcThrRS-D46E/H186G. F, post-transfer editing of Ser-tRNAThr by EcThrRS-D46E/Y173F (▲) and EcThrRS-D46E/H186G (▼). Spontaneous hydrolysis (●) and post-transfer editing of Ser-tRNAThr by EcThrRS (■) are included as negative and positive controls, respectively. The data in C, D, and F represent the averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.
control in the different Mycoplasma organisms. Notably, M. mobile encodes an editing-deficient LeuRS and PheRS, an evolutionary phenomenon that is probably advantageous, whereas it encodes an editing-capable ThrRS for the translation of Thr codons. Similarly, in M. capricolum, its ThrRS and PheRS are editing-deficient, whereas its LeuRS has been reported to be editing-capable (13). Thus, it is likely that different aaRSs require various levels of translational quality control at the codons of specific amino acids, even within a single Mycoplasma organism.

Experimental Procedures

Materials—L-Thr, 1-Ser, dithiothreitol, tetrasodium pyrophosphate, inorganic pyrophosphate, Tris-HCl, MgCl₂, NaCl, and activated charcoal were purchased from Sigma. [¹⁴C]Thr was obtained from Biotrend Chemicals (Destin, FL), and [¹⁴C]Ser and [α-³²P]ATP were obtained from PerkinElmer Life Sciences. DNA fragment rapid purification and plasmid extraction kits were purchased from Yuanpinghao Biotech (Tianjing, China). The KOD-plus mutagenesis kit was obtained from TOYOBO. T4 DNA ligase and restriction endonucleases were obtained from Thermo Scientific (Pittsburgh, PA). Phusion high fidelity DNA polymerase was purchased from New England Biolabs. Nickel-nitrotriacetic acid Superflow was purchased from Qiagen. Polyethyleneimine cellulose plates were purchased from Merck. Pyrophosphatase (PPIase) was obtained from Roche Applied Science. The dNTP mixture was obtained from TaKaRa (Japan). Oligonucleotide primers were synthesized by Invitrogen. E. coli BL21 (DE3) cells were purchased from Stratagene (Santa Clara, CA).

Cloning and Mutagenesis—The genomes of M. mobile and M. capricolum were kindly provided by Dr. Makoto Miyata (Osaka City University). The ORFs of the MmThrRS and McThrRS genes were amplified from genomic DNA and were separately inserted via restriction digestion into the pET28a (+) vector that was cut with Nhel-Xhol and Ncol-Xhol. All TGA codons (the codon encoding Trp in Mycoplasma species) in the ORFs of the MmThrRS and McThrRS genes were changed to Trp (TGG) codons (13). The recombinant plasmids containing the ScThrRS and EcThrRS genes, p425TEF-ScThrRS and p425TEF-EcThrRS, had been previously constructed in our lab (22). The ORFs encoding MmThrRS and McThrRS were inserted via restriction digestion into the yeast p425TEF expression vector that was cut with BamHI-Xhol to form p425TEF-MmThrRS and p425TEF-McThrRS, respectively. All genes with deletions and point mutations were made by following the instructions in the KOD-plus mutagenesis kit. All constructs were confirmed by DNA sequencing.

Gene Expression and Protein Purification—E. coli BL21 (DE3) cells were transformed with the recombinant plasmids and cultured in 2X YT (1.6% peptone, 1% yeast extract, and 0.5% NaCl) at 37 °C. Once the cultures had reached mid-log phase (A₆₀₀ = 0.6), gene expression was induced with 50 μM isopropyl-1-thio-β-D-galactopyranoside for 6 h at 18 °C. Protein purification was performed as previously described (32).

tRNA Gene Cloning, Expression, and Purification—According to our previous work, four DNA fragments covering the E. coli tRNAThr(UGU) gene double strands were synthesized by Invitrogen, phosphorylated, and ligated into pTrc99b (precleaved by PstI and EcoRI) to construct the pTrc99b-tRNAThr plasmid (33). E. coli MT102 cells were transformed with pTrc99b-tRNAThr and cultured to express the tRNAThr gene. The Thr accepting activity of the tRNAThr isolated from the extract of the transformants reached 1500 pmol/A₂₆₀. Because the accepting activity of pure tRNAThr was considered to be 1600 pmol/A₂₆₀, tRNAThr should comprise ~93% of the total tRNA.

³²P Labeling of tRNAThr—³²P labeling of the 3′-end of the tRNAThr was performed at 37 °C in a mixture containing 60 mM Tris-HCl (pH 8.0), 12 mM MgCl₂, 20 μM tRNAThr, 0.5 mM DT T, 20 μM ATP, 50 μM tetrasodium pyrophosphate, 0.666 μM [α-³²P]ATP, and 10 μM CCA-adding enzyme (CCase) for 5 min, as described (34). Finally, 0.8 unit/μl PPIase was added to the mixture for 2 min. [³²P]tRNAThr was extracted with phenol/chloroform twice, precipitated with ethanol, and dissolved in 5 mM MgCl₂.

ATP-PPI Exchange—In the ATP-PPI exchange reaction, the kinetic parameters of McThrRS or MmThrRS for Thr were measured at 30 °C in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 30 mM KCl, 2 mM DTT, 2.5 mM ATP, 2 mM tetrasodium [³²P]pyrophosphate, and 30–50,000 μM Thr. The reaction was initiated by the addition of either 200 nM McThrRS or 500 nM MmThrRS. For Ser, the reaction was performed at 30 °C in a similar reaction mixture containing Ser (62.5–1075 mM) and either McThrRS (500 nm) or MmThrRS (1 μM). At various time intervals, 15-μl aliquots of the reaction mixtures were taken and quenched to 200 μl with a solution containing 2% activated charcoal, 3.5% HClO₄, and 50 mM tetrasodium pyrophosphate. The solution was filtered through a Whatman GF/C filter and was then washed with 20 ml of 10 mM tetrasodium pyrophosphate solution and 10 ml of 100% ethanol. The filters were dried, and the [³²P]ATP was measured using a scintillation counter (Beckman Coulter).

Aminoaacylation—To generate time course curves of the aminoaacylation of tRNAThr catalyzed by McThrRS, McThrRS, and their derivatives, the reaction was performed at 30 °C in reaction buffer containing 60 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 30 mM KCl, 2 mM DTT containing 2.5 mM ATP, 10 μM tRNAThr, 285.5 μM [¹⁴C]Thr, and 200 nm enzymes. Time course curves of the aminoaacylation assays for tRNAThr by EcThrRS and its mutants were performed at 37 °C in a similar reaction buffer containing 114.2 μM [¹⁴C]Thr and 200 nm EcThrRS and its mutants. At various time intervals, 9-μl aliquots of the reaction solution were added to Whatman filter pads and quenched with cold 5% TCA. The pads were washed three times for 15 min each with cold 5% TCA and then three times for 10 min each with 100% ethanol. The pads were then dried under a heat lamp, and the radioactivity of the precipitates was quantified using a scintillation counter (Beckman Coulter).

In the ATP-PPI exchange reaction, the Kᵦᵦ values of MmThrRS and McThrRS for Thr (3.39 and 4.40 mM, respectively) were significantly higher than those of other ThrRSs (for example, 0.21 mM for ScThrRS) (22). In addition, the signal from [³²P]AMP or aminoacyl-[³²P]AMP could be directly observed on the plates used in the TLC assays (34). To improve the accuracy of the aminoaacylation assay, their kinetic parameters were measured using [³²P]tRNAThr. The kinetic parame-
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ters of McThrRS and McThrRS–ΔN1 were measured at 30 °C in a similar reaction mixture using 0.137 μM [32P]tRNAThr and 200 nm enzyme. For Thr, the concentration of Thr varied from 0.1–2 mM; for ATP, the concentration was changed from 0.005 mM to 1 mM; for tRNAThr, 0–20 μM cold tRNAThr was used. The kinetic parameters of EcThrRS and EcThrRS–ΔN1 were measured at 37 °C using 0.137 μM [32P]tRNAThr and 25 nm enzyme. For Thr, the concentration was varied from 5–2,000 μM, for ATP, the concentration was changed from 5 to 2000 μM, and for tRNAThr, 0–20 μM cold tRNAThr was used.

At specific time points, samples were taken for ethanol precipitation with NaAc (pH 5.2) at −20 °C overnight. The precipitated samples were centrifuged (10,000 × g) at 4 °C for 30 min, and dried at room temperature for 30 min, and digested with 6 μL of nucleosil S1 (25 units) for 2 h at 37 °C. After treatment with nucleosil S1, aminoacyl-[32P]AMP and [32P]AMP should be produced from aminoacyl-[32P]tRNA and free [32P]tRNA, respectively. Samples (2 μL) of the digestion mixture were loaded and separated by TLC in 0.1 M NH₄Ac and 5% acetic acid. The plates were utilized for phosphorimaging, and the data were analyzed using Multi-Gauge software (version 3.0 software (FUJIFILM)).

Misaminoacylation—Misaminoacylation by MmThrRS, McThrRS, and EcThrRS was assessed at 30 °C in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 30 mM KCl, 2.5 mM ATP, 0.2 μM [32P]tRNAThr, 10 μM cold tRNAThr, and either 1 mM Ser (for MmThrRS and McThrRS) or 100 μM Ser (for EcThrRS), plus 1 μM enzyme.

Post-transfer Editing—The post-transfer editing activity of the various ThrRSs and mutants was assessed by the hydrolysis of [14C]Ser-tRNAThr and was measured at 30 °C (for Mycoplasma ThrRSs) or 37 °C (for EcThrRSs) in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 30 mM KCl, 2 μM [14C]Ser-tRNAThr, and 200 nm enzyme. The preformed [14C]Ser-tRNAThr was prepared by using editing-deficient EcThrRS–H73A/H77A (21).

AMP Formation Assay—AMP formation was assayed by TLC at 30 °C in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 30 mM KCl, 10 units/ml PPhlase, 10 mM Thr or 1 mM Ser, 3 μM [α-32P]ATP, and 2 μM Mycoplasma ThrRSs or its mutant derivatives, either in the absence or presence of 20 μM tRNAThr. For EcThrRS and its mutants, the reaction was performed at 37 °C in the same reaction mixture but with the addition of 40 mM Ser and 1 μM enzyme. Samples (1.5 μL) were quenched in 6 μL of 200 mM NaAc (pH 5.0). The quenched aliquots (1.5 μL of each sample) were then spotted onto polyethyleneimine cellulose plates that had been preswathed with water. The separation of Ser-[α-32P]AMP, [α-32P]AMP, and [α-32P]ATP was performed in 0.1 M NH₄Ac and 5% acetic acid. The plates were visualized by phosphorimaging, and the data were analyzed using Multi Gauge software (version 3.0, FUJIFILM). Quantification of [α-32P]AMP was achieved by densitometry relative to samples containing known concentrations of [α-32P]ATP. The rates were obtained using only the initial time points, where the plot of [α-32P]AMP versus time was linear. The data were then fit to the following equation: y = b + k_{obs}t, where b and k represent the burst amplitude and the steady-state rate, respectively. The observed reaction rate constants (k_{obs}) were obtained by dividing the steady-state rate of the reaction by enzyme concentration.

Measurement of Dissociation Constants for tRNAThr by Filter Binding Assays—The formation of the EcThrRS–ΔN1/EcThrRS–[32P]tRNAThr complex was monitored using the nitrocellulose filter binding method (35). Nitrocellulose membranes (0.22 μm) were presoaked in washing buffer (50 mM Heps/KOH, pH 6.8, 50 mM KCl) for at least 10 min before use. The [32P]tRNAThr (27,353 c.p.m., 2.74 pmol) was incubated with either EcThrRS or EcThrRS–ΔN1 at various concentrations (0.2–9 μM) for 30 min at 0 °C in 50 mM of buffer containing 50 mM Heps/KOH (pH 6.8), 50 mM KCl and 12 mM MgCl₂. The samples were then applied and filtered through the nitrocellulose membrane. The filters were washed with 0.3 mL of washing buffer and dried, and the radioactivity was measured. The data were then analyzed using GraphPad Prism.

ScΔthrS Complementation Assay—The ScΔthrS yeast strain was constructed in our lab (22). For complementation assays, the genes were inserted into p425TEF, which was modified to have an in-frame DNA sequence encoding a His₆ tag at the C terminus of the ThrRSs. The constructs were transformed into ScΔthrS using the LiAc method (36). Transformants were selected on SD/Ura–/Leu–/G418 plates, and a single clone was cultured in liquid SD/Leu–/G418 medium. The culture was then diluted to a concentration equivalent to 1 A₆₀₀ and a 10-fold dilution of the yeast was plated onto SD/Leu–/G418 in the presence of 5-FOA to induce the loss of the rescue plasmid (pRS426-ScΔthrS). Complementation was observed by comparing the growth rates of ScΔthrS expressing native ScThrRS, MmThrRS, McThrRS, EcThrRS, and their mutants.

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References
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