Translational Quality Control by Bacterial Threonyl-tRNA Synthetases*

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Xiao-Long Zhou1,2, Yun Chen‡1, Zhi-Peng Fang‡, Zhi-Rong Ruan‡, Yong Wang5, Ru-Juan Liu3, Mei-Qin Xue‡, and En-Duo Wang3

From the 1State Key Laboratory of Molecular Biology, Chinese Academy of Sciences Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 200031 Shanghai, China and the 2School of Life Science and Technology, ShanghaiTech University, 200031 Shanghai, China

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)3 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 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 tRNALeu or tRNAPhe 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 amino acid activation and tRNA charging), and C-terminal (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 MmThrRS 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 EcThrRS is mediated by the communication between the N2 editing and N1 domains.

Results

Sequence Analysis of Mycoplasma ThrRSs—ThrRSs from bacteria (e.g., EcThrRS) 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 EcThrRS, 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 EcThrRS (including His73, His77, Lys156, Asp180, Cys182, and His186) (bold and ligh 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. MmThrRS 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 MmThrRS 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).

MmThrRS Is Editing-capable, Whereas McThrRS 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 McThrRS (with a degenerate editing domain) and MmThrRS (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

\[
DF = \left( \frac{k_{cat}}{K_m} \right)_{cognate \ amino \ acid} / \left( \frac{k_{cat}}{K_m} \right)_{noncognate \ amino \ acid}
\]

We purified MmThrRS and McThrRS to homogeneity (Fig. 1C). The kinetics of MmThrRS and McThrRS 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 McThrRS and MmThrRS, respectively) (Table 1), which are significantly larger than the acceptable threshold (1/3300) (26). Thus, we concluded that each of these enzymes misactivates noncognate Ser and therefore is likely to require an editing function to ensure the correct Thr-tRNA\textsuperscript{Thr} formation.

To directly monitor the editing activities of the two enzymes, we prepared Ser-tRNA\textsuperscript{Thr} by using the editing-deficient EcThrRS-H73A/H77A (21). We found that MmThrRS, but not McThrRS, was able to hydrolyze Ser-tRNA\textsuperscript{Thr} and remove noncognate Ser (Fig. 2A), thus indicating that the degeneration of the crucial editing active sites of McThrRS impairs its post-transfer editing. In contrast, MmThrRS, which has an intact N2 domain, has post-transfer editing activity. We further mutated two conserved His residues of the MmThrRS N2 domain (His\textsuperscript{9} and His\textsuperscript{13}), whose counterparts participate in the editing activities of EcThrRS (His\textsuperscript{73} and His\textsuperscript{77}) (21) and ScThrRS (His\textsuperscript{151} and His\textsuperscript{155}) (22). The MmThrRS-H9A/H13A mutant had aminoacylation activity (data not shown) similar to that of MmThrRS, but its post-transfer editing was not detected \textit{in vitro}, thus indicating that the N2 domain is involved in post-transfer editing (Fig. 2A).

Furthermore, the misaminoacylation of tRNA\textsuperscript{Thr} with Ser by McThrRS, MmThrRS, and EcThrRS (as an editing-capable control) was performed. We found that Ser-tRNA\textsuperscript{Thr} was apparently formed by McThrRS, whereas only trace amounts of Ser-tRNA\textsuperscript{Thr} were formed by MmThrRS, which was comparable with EcThrRS (Fig. 2, B and C).

These data indicate that MmThrRS is an editing-capable ThrRS, whereas the McThrRS has lost its post-transfer editing activity, probably because of its degenerate editing active site in the N2 domain. Thus, because it clearly synthesized mischarged tRNA\textsuperscript{Thr}, McThrRS is an error-prone tRNA synthetase.

MmThrRS and McThrRS Have Negligible tRNA-dependent Pretransfer Editing Capacities—In addition to their post-transfer editing activity, eukaryotic cytoplasmic ThrRSs (e.g., ScThrRS) also exhibit tRNA-independent and tRNA-dependent pretransfer editing activities, contributing the highest pro-

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portion in the overall editing pathways (22). 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 McThrRS 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 MmThrRS were $0.224 \pm 0.027$ and $0.225 \pm 0.032$ s$^{-1}$, respectively, which were larger than those for McThrRS (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. 4, A), 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 tRNA\textsuperscript{Thr} was derived from tRNA-independent pretransfer editing but not misactivation of Ser and then the subsequent hydrolysis of Ser-AMP. Ser-AMP formation by Mc\textsubscript{ThrRS} was more apparent than that of Mm\textsubscript{ThrRS} in the absence (0.020 ± 0.005 and 0.068 ± 0.018 s\textsuperscript{-1} for Mm\textsubscript{ThrRS} and Mc\textsubscript{ThrRS}, respectively) or presence (0.008 ± 0.002 and 0.072 ± 0.028 s\textsuperscript{-1} for Mm\textsubscript{ThrRS} and Mc\textsubscript{ThrRS}, respectively) of tRNA (Fig. 3, A and B, and Table 2). Furthermore, the \( k_{\text{obs}} \) value of AMP formation of post-transfer editing-defective Mm\textsubscript{ThrRS-H9A/H13A} was 0.091 ± 0.017 s\textsuperscript{-1} in the presence of tRNA\textsuperscript{Thr}, which was comparable with that of Mm\textsubscript{ThrRS}. The \( k_{\text{obs}} \) value of Ser-

**TABLE 2**

\( k_{\text{obs}} \) values of Mm\textsubscript{ThrRS}, Mm\textsubscript{ThrRS-H9A/H13A}, and Mc\textsubscript{ThrRS} in AMP and Ser-AMP formation

The results are the averages of three independent repeats, with the standard deviations indicated.

| Enzyme          | tRNA | AMP formation | Ser-AMP formation |
|-----------------|------|---------------|-------------------|
| Mm\textsubscript{ThrRS} | -    | 0.093 ± 0.011 | 0.020 ± 0.005     |
|                 | +    | 0.086 ± 0.010 | 0.008 ± 0.002     |
| Mm\textsubscript{ThrRS-H9A/H13A} | +    | 0.091 ± 0.017 | 0.021 ± 0.007     |
| Mc\textsubscript{ThrRS}       | -    | 0.224 ± 0.027 | 0.068 ± 0.018     |
|                 | +    | 0.225 ± 0.032 | 0.072 ± 0.028     |
AMP formation of MmThrRS-H9A/H13A was 0.021 ± 0.007 s⁻¹ in the presence of tRNA₅⁰Thr, which was larger than that of MmThrRS (Table 2). These data clearly show that tRNA₅⁰Thr 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 (ScΔthrS) as an in vivo tool to assess the functions of various ThrRSs (22). To determine whether MmThrRS and McThrRS could complement the ScΔthrS and to evaluate the effects of defective editing of ThrRS on yeast phenotypes, we introduced the McThrRS and MmThrRS genes into ScΔthrS and observed the growth of the transformants on selective medium. Interestingly, only MmThrRS was able to rescue the ScΔthrS phenotypes in the presence of 5-fluoroorotic acid (5-FOA) (Fig. 5A). The inability of the McThrRS to rescue ScΔthrS growth might have originated from its failure to aminoacylate yeast tRNA₅⁰Thr isoacceptors or to perform post-transfer editing. To distinguish between these two possibilities, we assayed the aminocytlation of the three yeast isoacceptors tRNA₅⁰Thr (UGU), tRNA₅⁰Thr (AGU), and tRNA₅⁰Thr (AGU) by McThrRS. Our data showed that McThrRS aminoacylated each yeast tRNA₅⁰Thr 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 (Met¹–Gly²¹⁴) with the N2 domain of MmThrRS (Met¹–Gly¹⁵⁷) to obtain the chimeric McThrRS-MmN2-DN1 (without N1) (Fig. 5C). McThrRS-MmN2-DN1 had an aminoacylation activity comparable with that of McThrRS (data not shown). An in vitro hydrolytic assay of Ser-tRNA₅⁰Thr showed that the translocation of the N2 domain of MmThrRS restored the post-transfer editing capability of McThrRS (Fig. 5D). Furthermore, the chimeric enzyme in ScΔthrS 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 ScΔthrS. Mutations at these two editing-critical 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-critical residues (including Lys⁸⁶, Asp¹¹⁷, Cys¹¹⁹, and His¹²³) (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 ScΔthrS 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, McThrRS-H9A/H13A, and McThrRS-N2M in ScΔthrS on the basis of a His₆ tag at the C terminus of each protein (Fig. 5G) or the in vitro aminoacylation of tRNA₅⁰Thr 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 ScΔthrS.

These observations suggest a correlation between the growth phenotype of ScΔthrS and the editing capacities of various native or mutant ThrRSs (Fig. 5C). The findings also clearly show that ScΔthrS cells are highly sensitive to post-transfer editing catalyzed by introduced bacterial ThrRSs. Therefore, ScΔthrS 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 (Met¹–Leu⁶⁷) at the N terminus of MmThrRS, resulting in the chimeric mutant MmThrRS–ΔMcN1 (Fig. 5C). MmThrRS–ΔMcN1 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–ΔMcN1 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–ΔMcN1 expression level was comparable with that of ScΔthrS (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 ScThrS loss of function yeast. A, complementation of an ScThrS loss of function mutant strain by MmThrRS, MmThrRS-H9A/H13A, and McThrRS. ScThrS and p425TEF are used as positive and negative controls, respectively. B, aminoacylation of yeast tRNA^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 ScThrS (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^Thr^ by the McThrRS-MmN2-ΔN1 (■) and McThrRS-N2M (□). E, rescue of the ScThrS loss-of-function strain by McThrRS, McThrRS-MmN2 and McThrRS-MmN2-ΔN1. EcThrRS and p425TEF are shown as positive and negative controls, respectively. F, rescue of the ScThrS 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_6_ tag at the C terminus. ScThrS expressed from a rescue plasmid had no His_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 McThrRS-ΔN1 from the E. coli transformants because of the formation of inclusion bodies. Similarly, after the replacement of the McThrRS N2 domain (Asn<sup>68</sup>–Gly<sup>214</sup>) with its McThrRS equivalent (Asn<sup>8</sup>–Gly<sup>157</sup>), the chimeric McThrRS-MmN2 formed inclusion bodies in the E. coli transformants (data not shown) and was unable to rescue ScΔthrS growth (Figs. 5C and 6D). However, McThrRS-MmN2-ΔN1 was functional both in <i>in vitro</i> and <i>in vivo</i> (Fig. 5, D and E). The phenotypic differences between the McThrRS and MmThrRS-ΔN1 or McThrRS-MmN2 and McThrRS-MmN2-ΔN1 (only differing in the absence or presence of the N1 domain of McThrRS in each pair) (Fig. 5C) imply that the functional N2 domain of McThrRS is incompatible with the N1 domain of McThrRS. Together, the above data suggest that the absence of an N1 domain in McThrRS increases its catalytic efficiency and stability.

The N1 Domain Is Essential for Editing by EcThrRS—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 McThrRS (Fig. 1A). Although functions have been assigned to the other ThrRS 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<sup>1</sup>–Asn<sup>58</sup>) of McThrRS to obtain the deletion mutant McThrRS-ΔN1 (Lys<sup>59</sup>–Lys<sup>639</sup>) (Fig. 5C). As expected, McThrRS-ΔN1 did not rescue the growth of ScΔthrS (Figs. 5C and 6D). Recombinant McThrRS-ΔN1 was successfully purified to high homogeneity from <i>E. coli</i> transformants (Fig. 1C), and its kinetic constants in the aminoacylation reaction for three substrates were measured. As compared with those of McThrRS, the <i>K<sub>m</sub></i> values of McThrRS-ΔN1 for Thr and ATP were little changed, whereas the <i>K<sub>m</sub></i> value for tRNA<sup>Thr</sup> was decreased (2.00 ± 0.28 of McThrRS versus 0.94 ± 0.17 μM of McThrRS-ΔN1). The <i>k<sub>cat</sub></i> values for the three substrates were marginally decreased after truncation. Overall, there was little change in the enzymatic activities after the deletion of the McThrRS N1 domain (Table 3). As anticipated, McThrRS-ΔN1 was deficient in post-transfer editing similar with native McThrRS (data not shown).

Because of the editing deficiency of McThrRS and its inability to support the growth of ScΔthrS, we were unable to assess the contribution of the N1 domain to the editing activity of ThrRS and its <i>in vivo</i> function. Therefore, we further used EcThrRS, which has editing function (21) and was able to replace yeast ThrRS for <i>in vivo</i> aminoacylation (Fig. 6A). An EcThrRS-ΔN1 (Lys<sup>63</sup>–Glu<sup>642</sup>) expression vector was constructed, and the enzyme was purified (Fig. 1C). Consistently with the <i>in vitro</i> data of aminoacylation kinetics from McThrRS and McThrRS-ΔN1 (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<sub>d</sub>) values of the native EcThrRS and EcThrRS-ΔN1 with tRNA<sup>Thr</sup> by using a filter-binding assay. The k<sub>d</sub> values for EcThrRS and EcThrRS-ΔN1 were 1.92 ± 0.23 and 1.54 ± 0.20 μM, respectively, indicating that the N1 truncation has a negligible effect on the protein structure (Fig. 7A). However, the EcThrRS-ΔN1 enzyme did not support the growth of ScΔthrS (Figs. 5C and 7B). Thus, we speculated that editing is impaired in EcThrRS-ΔN1. Indeed, the hydrolisis of Ser-tRNA<sup>Thr</sup> revealed that EcThrRS-ΔN1 had no post-transfer editing (Fig. 7C). The editing deficiency and failure to rescue ScΔthrS containing EcThrRS-ΔN1 were reminiscent of the characteristics of McThrRS and MmThrRS-N2M, thus strongly suggesting that ScΔthrS cell viability is directly regulated by the ThrRS editing function. Both McThrRS and McThrRS lack tRNA-dependent pretransfer editing capacity. To elucidate whether EcThrRS has tRNA-independent and tRNA-dependent pretransfer editing activities, along with any potential role of the N1 domain, we assayed AMP formation catalyzed by EcThrRS and EcThrRS-ΔN1, respectively. The amount of AMP produced by EcThrRS through a TLC-based editing assay was significantly stimulated in the presence of tRNA (Fig. 7, D and E), and the calculated k<sub>obs</sub> values were 1.81 ± 0.21 s<sup>−1</sup> with tRNA and 0.33 ± 0.05 s<sup>−1</sup> without tRNA, thus indicating strong tRNA-dependent editing, including the pre- and post-transfer editing of EcThrRS (Table 5). However, under the same conditions, the k<sub>obs</sub> values of the EcThrRS-ΔN1 in the presence and absence of tRNA<sup>Thr</sup> were only slightly different (0.34 ± 0.06 s<sup>−1</sup> with tRNA and 0.25 ±

![FIGURE 6. Addition of the N1 domain of McThrRS is detrimental for McThrRS function. A, yeast complementation assay with different ThrRSs and McThrRS-ΔN1. B, steady-state protein levels of McThrRS, McThrRS-ΔN1, and EcThrRS. GAPDH is included as a loading control. A nonspecific band is indicated with an asterisk. C, McThrRS, McThrRS-ΔN1, and EcThrRS relative protein levels after calculation of the normalized ratio of ThrRSs to GAPDH. The data in C represent averages of three independent experiments and the corresponding standard errors. D, yeast complementation by McThrRS-MmN2 and McThrRS-ΔN1.](image-url)
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 $\text{Mm}$$\text{ThrRS}$ and $\text{Mc}$$\text{ThrRS}$ (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-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 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 $\text{Ec}$$\text{ThrRS}$, we subsequently studied its mechanism. The editing active site of the ThrRS is within the N2 domain; however, our above data showed that the N1 domain is involved in the editing activity of ThrRS. The N1 domain is located at the N terminus of the N2 editing domain, far away from the mischarged tRNA$^\text{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 Asp$^{46}$ 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) Asp$^{46}$ 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 Asp$^{46}$ in the in vivo function of EcThrRS, Asp$^{46}$ 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 Asp$^{46}$ mutants were able to rescue the growth of the thrS deletion strain (Fig. 8C), including the Glu$^{46}$ 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 Asp$^{46}$ is located in the N1 domain, it is likely that Asp$^{46}$ 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 Asp$^{46}$ potentially interacts with the side chain of Lys$^{136}$ (2.95 Å) or Tyr$^{173}$ (2.66 Å) in the N2 domain, whereas another oxygen atom of Asp$^{46}$ likely interacts with the amide group of the His$^{186}$ main chain (2.83 Å) in the N2 domain (Figs. 8B and 9A). The Lys$^{136}$ 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 Asp$^{46}$ 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 Glu$^{136}$ and Asp$^{46}$ into close proximity, inducing structural disruption, thus accounting for the failure of EcThrRS-K136E to rescue growth of ScΔthrS. In contrast, Ala$^{136}$ in EcThrRS-K136A and Arg$^{136}$ in EcThrRS-K136R are accommodated both in terms of size (a small methyl group) or complementary charge. After substitution of Tyr$^{173}$ with Ala, Asp, or Arg, none of the three mutants, EcThrRS-Y173A, -Y173R, or -Y173D, rescued the growth of ScΔthrS, thus implying that Tyr$^{173}$ 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 Asp$^{46}$ in the N1 domain and the Tyr$^{173}$ 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, Asp$^{46}$ potentially also interacts with the main chain amide group of His$^{186}$. When His$^{186}$ 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

| Enzyme     | Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|------------|-----------|----------|-------|--------------|
| EcThrRS    | Thr       | 0.69 ± 0.08 | 108.33 ± 11.24 | 6.37         |
| ATP        | 0.63 ± 0.09 | 36.71 ± 2.97 | 17.16   |
| EcThrRS-ΔN1| Thr       | 0.71 ± 0.07 | 0.80 ± 0.11 | 887.50      |
| ATP        | 0.64 ± 0.06 | 168.02 ± 18.61 | 3.81   |
| tRNA$^\text{Thr}$ | 0.63 ± 0.05 | 35.17 ± 4.18 | 17.91   |
| tRNA$^\text{Thr}$ | 0.58 ± 0.05 | 0.72 ± 0.15 | 805.56   |

### Proofreading by Various Threonyl-tRNA Synthetases

Because Asp$^{46}$ is located in the N1 domain, it is likely that Asp$^{46}$ interacts with the main chain amide group of the His$^{186}$. When His$^{186}$ was substituted with Gly (EcThrRS-H186G) to minimize the side chain and thereby enhance the flexibility, the EcThrRS-H186G complemented ScThrRS very well.
Proofreading by Various Threonyl-tRNA Synthetases

![Graph A: k<sub>c</sub> determinations for EcThrRS (●) and EcThrRS-ΔN1 (■) for tRNA<sub>Thr</sub>.](image1)

**FIGURE 7. Role of N1 domain of EcThrRS.** A, k<sub>c</sub> determinations for EcThrRS (●) and EcThrRS-ΔN1 (■) for tRNA<sub>Thr</sub>. B, 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<sub>Thr</sub> by EcThrRS (●) and EcThrRS-ΔN1 (■). The spontaneous hydrolysis of Ser-tRNA<sub>Thr</sub> (●) is included as a negative control. D–G, generation of [32P]AMP in the absence (+tRNA) or presence (+−tRNA) of tRNA<sub>Thr</sub> 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 mM) 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<sub>obs</sub> values of EcThrRS and EcThrRS-ΔN1 in AMP formation assays

| Enzyme       | tRNA | k<sub>obs</sub> (s<sup>-1</sup>) |
|--------------|------|---------------------------------|
| EcThrRS      | −    | 0.33 ± 0.05                     |
|              | +    | 1.81 ± 0.21                     |
| EcThrRS-ΔN1  | −    | 0.25 ± 0.04                     |
|              | +    | 0.34 ± 0.06                     |

The results are the averages of three independent repeats, with the standard deviations indicated.

(data not shown). Interestingly, when Gly<sup>186</sup> 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<sup>186</sup>. 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<sup>46</sup> in the N1 domain and Lys<sup>136</sup>, Tyr<sup>173</sup>, and His<sup>186</sup> 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<sup>46</sup> and Tyr<sup>173</sup> is important. However, EcThrRS-D46E/Y173F was functional in supporting yeast growth; it should have no hydrogen bond between Glu<sup>46</sup> and Phe<sup>173</sup> 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. Interestingly, EcThrRS-D46E/H186G supports growth of Sc/hr9004thrs 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 Sc/hr9004thrs was more sensitive than with the in vitro hydrolysis of Ser-tRNAth nam. Hence, Sc\(\Delta\)hrTS 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-\(\Delta\)N1, 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 inter-domain 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 ThrRS.
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 CCA_{76} 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, similar 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 Asp^{46}, Lys^{136}, Tyr^{173}, and His^{186}. The side chains of the Asp^{46}, Lys^{136}, and Tyr^{173} residues and the main chain of the His^{186} residue are shown as sticks (left) and spheres (right). The distance between Asp^{46} and the other residues is indicated. B, yeast complementation by mutants of the Lys^{136} and Tyr^{173} 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-tRNA^{Thr} by EcThrRS-Y173R (□) and EcThrRS-Y173D (△). Spontaneous hydrolysis (○) and post-transfer editing of Ser-tRNA^{Thr} 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-tRNA^{Thr} by EcThrRS-D46E/Y173F (▲) and EcThrRS-D46E/H186G (▼). Spontaneous hydrolysis (○) and post-transfer editing of Ser-tRNA^{Thr} 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, t-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 TaKaRa (Japan). Oligonucleotide primers were obtained from Roche Applied Science. The dNTP mixture was purchased from Merck. Pyrophosphatase (PPiase) was purchased from Qiagen. Polyethyleneimine cellulose plates were purchased from Sisco Research Laboratory (Mumbai, India). Nickel-nitrilotriacetic acid Superflow was purchased from Thermo Fisher Scientific (Pittsburgh, PA). Phusion high fidelity DNA polymerase was purchased from New England Biolabs. Nickel-nitrilotriacetic 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 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 Mm ThrRS 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 TGG codons (13). The recombinant plasmids containing the StThrRS and EcThrRS genes, p425TEF-StThrRS 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 2 X 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 tRNA^{Thr}(UGU) gene double strands were synthesized by Invitrogen, phosphorylated, and ligated into pTrc99b (precleaved by PstI and EcoRI) to construct the pTrc99b-tRNA^{Thr} plasmid (33). E. coli MT102 cells were transformed with pTrc99b-tRNA^{Thr} and cultured to express the tRNA^{Thr} gene. The Thr accepting activity of the tRNA^{Thr} isolated from the extract of the transformants reached 1500 pmol/A₂₆₀. Because the accepting activity of pure tRNA^{Thr} was considered to be 1600 pmol/A₂₆₀, tRNA^{Thr} should comprise ~93% of the total tRNA.

**³²P Labeling of tRNA^{Thr}**—³²P labeling of the 3’-end of the tRNA^{Thr} was performed at 37 °C in a mixture containing 60 mM Tris–HCl (pH 8.0), 12 mM MgCl₂, 20 μM tRNA^{Thr}, 0.5 mM DTT, 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]tRNA^{Thr} 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, 5 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).

**Aminoaoylation**—To generate time course curves of the aminoaoylation of tRNA^{Thr} 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 tRNA^{Thr}, 285.5 μM [³¹C]Thr, and 200 nM enzymes. Time course curves of the aminoaoylation assays for tRNA^{Thr} by EcThrRS and its mutants were performed at 37 °C in a similar reaction buffer containing 114.2 μM [³¹C]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 Kₚ 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 StThrRS) (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 aminoaoylation assay, their kinetic parameters were measured using [³²P]tRNA^{Thr}. The kinetic paramet-
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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 enzymes. 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 5–200 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, dried at room temperature for 30 min, and digested with 6 μL of nuclease S1 (25 units) for 2 h at 37 °C. After treatment with nuclease 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 0.1M NH4Ac and 5% acetic acid. The plates were visualized by phosphorimaging, and the data were then fit to the following equation:

\[
\frac{\text{obs}}{\text{lof}} = \frac{\text{burst}}{\text{lof}} + \frac{1}{\text{obs} / \text{lof}}
\]

The kinetic parameters of ThrRSs or 37 °C (for ThrRSs) 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 mM 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 μL/mL PPlase, 10 mM Thr or 1 mM Ser, 3 mM [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 prewashed 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 $k_{obs}$ 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 HEPES/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 μL of buffer containing 50 mM HEPES/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-ScthrS). Complementation was observed by comparing the growth rates of ScΔthrS expressing native ScThrRS, MmThrRS, McThrRS, EcThrRS, and their mutants.

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