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Identification of determinants for tRNA substrate recognition by *Escherichia coli* C/U34 2′-O-methyltransferase

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Post-transcriptional modifications bring chemical diversity to tRNAs, especially at positions 34 and 37 of the anticodon stem-loop (ASL). TrmL is the prokaryotic methyltransferase that catalyzes the transfer of the methyl group from S-adenosyl-L-methionine to the wobble base of $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{UAA}$ isoacceptors. This Cm34/Um34 modification affects codon-anticodon interactions and is essential for translational fidelity. TrmL-catalyzed 2′-O-methylation requires its homodimerization; however, understanding of the tRNA recognition mechanism by TrmL remains elusive. In the current study, by measuring tRNA methylation by TrmL and performing kinetic analysis of tRNA mutants, we found that TrmL exhibits a fine-tuned tRNA substrate recognition mechanism. Anticodon stem-loop minihelices with an extension of 2 base pairs are the minimal substrate for *Ec*TrmL methylation. A35 is a key residue for TrmL recognition, while A36-A37-A38 are important either via direct interaction with TrmL or due to the necessity for prior isopentenylation (i^6) at A37. In addition, TrmL only methylates pyrimidines but not purine residues at the wobble position, and the 2′-O-methylation relies on prior N^6-isopentenyladenosine modification at position 37.

Introduction

Transfer RNAs (tRNAs) act as adaptors by linking nucleotide sequences and amino acids through codon-anticodon pairing.[1] tRNAs function only following post-transcriptional modifications during tRNA maturation.[2] Modifications to tRNAs are the most extensive of all classes of RNA molecules.[3] tRNA post-transcriptional modifications are universally found in bacteria, archaea and eukarya.[4] More than 90 modifications have been identified in tRNA nucleotides,[5,6] with the majority occurring in the main body and the anticodon stem loop (ASL), especially at positions 34 and 37.[2,7] Modifications in the anticodon region have been shown to be the most effective to restrict the motional dynamics of tRNAs.[8] Many of these modifications contribute to efficiency and fidelity of protein synthesis, and are further involved in the cellular stress response, the immune response and even human diseases such as cancers, neurological disorders, diabetes, and many mitochondrial disorders.[9-14] A notable example is the Trm9-mediated mcm^5U modification of the tRNA wobble base U34 in *Saccharomyces cerevisiae*, which enhances codon-specific translation elongation and modulates expression levels of critical damage response proteins during the cellular DNA damage response.[15]

2′-O-methylation of wobble nucleotide 34 in the 2 *Escherichia coli* isoacceptors $tRNA^{Leu}_{CAA}$ (*Ec*$tRNA^{Leu}_{CAA}$) and $tRNA^{Leu}_{UAA}$ (*Ec*$tRNA^{Leu}_{UAA}$) is incompletely characterized.[16] The 2′-O-methylation of pyrimidine nucleotides at position 34 can stabilize the C3′-endo form of the 3′-nucleotidyl unit to confer local conformation rigidity.[17] Deficient 2′-O-methylation in *E. coli* results in reduced efficiency of codon-wobble base interactions and impacts the recovery of cells from the stationary phase.[16] The methyltransferase introducing the 2′-O-methyl group onto the 2 tRNA isoacceptors has been identified as a SPOUT (SpoU-TrmD) class member, TrmL.[16] The SPOUT superfamily is a class of S-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTases) with a characteristic α/β knot structure.[18,19] TrmL, which consists only of a SPOUT catalytic domain without the usual RNA binding extension domain, is the simplest MTase in the SPOUT family. Interestingly, TrmL can independently catalyze the transfer of the methyl group from the methyl donor SAM to the $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{UAA}$ isoacceptors without the aid of any other tRNA binding proteins in a direct methyltransfer assay using recombinant TrmL protein.[20]

The $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{UAA}$ isoacceptors are the only 2 RNA substrates of TrmL.[16,20] TrmL functions as a homodimer

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using basic amino acid surface residues of TrmL for tRNA recognition.[20] In prokaryotes, 3 other SPOUT tRNA MTases (TrmH, TrmD and TrmJ) exist; these SPOUT MTases have far more tRNA substrates than TrmL. TrmH catalyzes the 2′-O-methylation of tRNA G18.[21-24] Substitution at G18G19 causes deficiencies in methyl transfer activity, and the oxygen 6 atom of G18 is a key recognition element for TrmH.[25-27] TrmD and TrmJ act on the tRNA anticodon loop. TrmD is a tRNA (m^1G37) MTase, [28-32] containing an extension sequence for tRNA binding, in contrast to TrmL.[20] While G36G37 is the substrate determinant of TrmD, TrmD requires only an anticodon stem-loop structure with an extended stem.[28,31,33-37] TrmJ is the tRNA Um32/Cm32 methyltransferase.[38,39] *E. coli* TrmJ (*Ec*TrmJ) requires full-length tRNA molecules as substrates.[39] The elements of tRNA required for recognition and methylation by TrmL have not yet been identified. The mechanism by which TrmL localizes to the anticodon domain of the large L-shaped structure and distinguishes C/U in the wobble position from the other nucleotides is an intriguing question. In this study, we identified the essential nucleotides in $tRNA^{Leu}$ required for recognition by TrmL. In addition, TrmL activity is sensitive to the modified base at position 37, supporting the previous observation that a defect in synthesis of ms^2i^6A37 at position 37, led to the loss of 2′-O-methylation at C/U 34 at $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{UAA}$ isoacceptors.[16] The isopentenyl modification at position 37 of transcribed $tRNA^{Leu}$ is sufficient to recruit TrmL for methylation of nucleotides C34/U34.

Results

i^6A37 is sufficient to restore 2′-O-methylation at C34 of *Ec*$tRNA^{Leu}_{CAA}$ transcripts *in vitro*

It has previously been shown that *in vitro* tRNA transcripts lacking natural post-transcriptional modifications are not modified by *Ec*TrmL. [20] This suggests that one or more modifications that occur during the tRNA maturation process are essential for guiding *Ec*TrmL methylation activity. Earlier studies showed that the ms^2i^6 modification, in which MiaA catalyzes the transfer of 5-carbon dimethylallyl to the adenosine at position 37 in the first step, was the key modification essential for *Ec*TrmL activity.[16] We overexpressed and purified *Ec*$tRNA^{Leu}_{CAA}$ lacking the ms^2i^6 modification in a MiaA gene deletion strain (dMiaA-$tRNA^{Leu}_{CAA}$). Then, the ms^2i^6A37 deficient *Ec*$tRNA^{Leu}_{CAA}$ was tested in a tRNA methyl transfer assay. As anticipated from previous studies, [16] the ms^2i^6A37 deficient *Ec*$tRNA^{Leu}_{CAA}$ was not methylated at all by *Ec*TrmL (**Fig. 1A**); however, when the unmodified A37 base was modified *in vitro* by recombinant *Ec*MiaA, it became a substrate of *Ec*TrmL (**Fig. 1A**). *In vitro* synthesized *Ec*$tRNA^{Leu}_{CAA}$ transcript, modified by recombinant MiaA protein (i^6A-Ts-$tRNA^{Leu}_{CAA}$) was also used in the tRNA methyl transfer assay. The modified transcript (i^6A-Ts-$tRNA^{Leu}_{CAA}$) could be equally well methylated to modified $tRNA^{Leu}_{CAA}$ overexpressed from MiaA gene knockout strain (i^6A-dMiaA-$tRNA^{Leu}_{CAA}$) by *Ec*TrmL (**Fig. 1B**), suggesting that i^6A modification at position 37 is essential for guiding *Ec*TrmL methylation activity, consistent with the previous report that ms^2i^6A at position 37 is a prerequisite for TrmL activity. [16]

We next analyzed the binding affinities of *Ec*$tRNA^{Leu}_{CAA}$ with *Ec*TrmL using an electrophoretic mobility shift assay (**Fig. 1C**). Three tRNAs, including native *Ec*$tRNA^{Leu}_{CAA}$ extracted from strain JW3581-1 and *Ec*$tRNA^{Leu}_{CAA}$ transcript with or without the i^6A37 modification, were incubated with increasing concentrations of *Ec*TrmL (0.75–10 μM). For native *Ec*$tRNA^{Leu}_{CAA}$, the shift representing the *Ec*TrmL-tRNA complex (tRNA bound 1) was initially observed at an enzyme concentration of 0.75 μM. A supershift was observed above 3.0 μM *Ec*TrmL, representing a larger molecular mass complex or aggregate (tRNA bound 2). Transcripts of *Ec*$tRNA^{Leu}_{CAA}$ without any natural post-transcriptional modifications showed no detectable binding to *Ec*TrmL in the range of enzyme concentration tested. The assay with i^6A37 modified *Ec*$tRNA^{Leu}_{CAA}$ transcripts showed a decreased binding affinity to *Ec*TrmL when compared with native *Ec*$tRNA^{Leu}_{CAA}$. The shift was first observed at 1.5 μM enzyme, and a supershift was

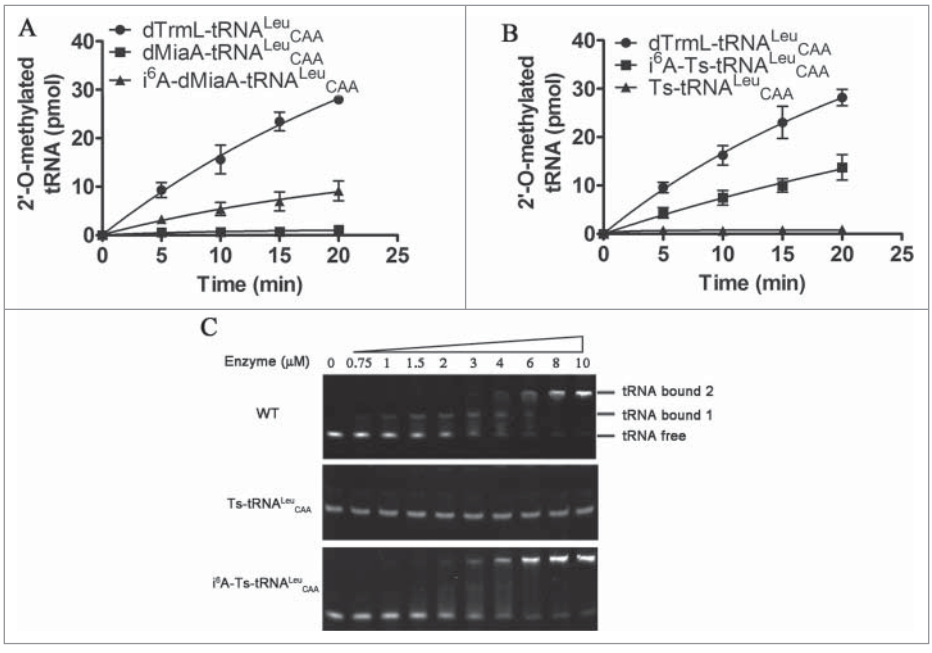

Figure 1. i^6A37 is essential for TrmL recognition. (**A**) The capacity of dMiaA-*Ec*$tRNA^{Leu}_{CAA}$ and i^6A-dMiaA-*Ec*$tRNA^{Leu}_{CAA}$ to be methylated by *Ec*TrmL. dTrmL-$tRNA^{Leu}_{CAA}$ was purified from a TrmL gene knockout strain and used as a positive control, and dMiaA-*Ec*$tRNA^{Leu}_{CAA}$ was purified from a MiaA gene knockout strain. (**B**) The capacity of i^6A-Ts-*Ec*$tRNA^{Leu}_{CAA}$ to be methylated by *Ec*TrmL. Ts-*Ec*$tRNA^{Leu}_{CAA}$ was transcribed by T7 RNA polymerase *in vitro* and used as a negative control. (**C**) The binding affinities of i^6A-Ts-*Ec*$tRNA^{Leu}_{CAA}$ to *Ec*TrmL as determined by electrophoretic mobility shift assay. The results are the average of 3 independent experiments with standard deviations indicated.

observed above 3.0 μM *Ec*TrmL. This assay showed that although the affinity of i^6A-Ts-tRNA$^{Leu}_{CAA}$ to *Ec*TrmL was lower than for WT *Ec*tRNA$^{Leu}_{CAA}$, the i^6A modification had a consequent effect on the binding strength of tRNA transcripts for *Ec*TrmL.

Construction of *Ec*tRNA$^{Leu}_{CAA}$ mutants

SPOUT MTase TrmL functions as homodimer by using basic surface amino acid residues to recognize its tRNA substrates. Only tRNA$^{Leu}_{CAA}$ and tRNA$^{Leu}_{UAA}$ isoacceptors are substrates for TrmL, however, nucleotides that determine the "TrmL identity" have not yet been determined. [16] We designed a systematic approach to replace nucleotides in *Ec*tRNA$^{Leu}_{CAA}$. **Figure 2A** shows the secondary structure of *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$. The two anticodon loops are conserved except for nucleotide 34, which are C in *Ec*tRNA$^{Leu}_{CAA}$ and U in *Ec*tRNA$^{Leu}_{UAA}$. The anticodon stem and variable loop do not show sequence conservation, especially within the variable loop region where *Ec*tRNA$^{Leu}_{UAA}$ has a longer stem than *Ec*tRNA$^{Leu}_{CAA}$. Based on these variations, we performed site-directed mutagenesis in *Ec*tRNA$^{Leu}_{CAA}$ as follows: deletions within the whole variable arm, which is consistent with most *Ec*tRNAs with the exception of *Ec*tRNA^{Ser} and *Ec*tRNA^{Tyr} (designated VLAD); deletion of the U-A base pair at position 46-47F in the variable arm (VLSD); and a guanine nucleotide insertion into the middle of variable loop between positions 47B and 47C (VLI). In the anticodon stem, we substituted the G-C base pair at position 30-40 to A-U (ASL30), and the adjacent A-U at position 31-39 to G-C (ASL31). In the anticodon loop, we substituted each nucleotide from positions 32 to 38 by changing purines to pyrimidines or *vice versa*, resulting in *Ec*tRNA$^{Leu}_{CAA}$-U32C/A/G, *Ec*tRNA$^{Leu}_{CAA}$-U33A, *Ec*tRNA$^{Leu}_{CAA}$-A35G/U/C, *Ec*tRNA$^{Leu}_{CAA}$-A36C, *Ec*tRNA$^{Leu}_{CAA}$-A37C, and *Ec*tRNA$^{Leu}_{CAA}$-A38C (**Fig. 2B**). Most of these tRNAs were overexpressed and purified by phenol extraction followed by DEAE-Sepharose chromatography and C18 reversed-phase HPLC as described below (see Materials and Methods). The tRNA mutants which could not be overexpressed in the TrmL gene deletion strain were transcribed by T7 RNA polymerase and modified by *Ec*MiaA at position 37 *in vitro*. As a control, wild type *Ec*tRNA$^{Leu}_{CAA}$ was purified in the TrmL gene deletion strain (dTrmL-*Ec*tRNA$^{Leu}_{CAA}$) or transcribed by T7 RNA polymerase *in vitro* (Ts-*Ec*tRNA$^{Leu}_{CAA}$).

The variable arm of tRNA is not a recognition element for *Ec*TrmL

*Ec*tRNA^{Leu}s belong to the small family of class II tRNAs which have more than 10 nucleotides in the variable arm. The long variable arm protrudes on one side of the tRNA and may affect binding or recognition by modification enzymes, or give information on how *Ec*TrmL approaches the wobble position of *Ec*tRNA. To address this possibility, we examined the effect of drastic mutations in the variable arm and loop of *Ec*tRNA$^{Leu}_{CAA}$ (mutants VLSD, VLAD and VLI, **Fig. 2B**). The methyl transfer assay showed that the 3 tRNA mutants were all methylated as well as the WT *Ec*tRNA$^{Leu}_{CAA}$ (dTrmL-*Ec*tRNA$^{Leu}_{CAA}$) (**Fig. 3A**). The steady-state kinetic constants shown in **Table 1** indicated that deletions of one base pair in the variable arm (VLSD), deletion of the entire variable arm (VLAD) or nucleotide insertions (VLI) had no significant effect on the methylation parameters, indicating that *Ec*TrmL is not sensitive to either the sequence or the length of tRNA variable loop.

The anticodon stem of tRNA does not contain recognition elements for *Ec*TrmL

Sequence comparison of the 2 *Ec*tRNA^{Leu} isoacceptors showed that the fourth and fifth base pairs in the anticodon stem of the 2 *Ec*tRNA^{Leu} isoacceptors are conserved (**Fig. 2A**). We performed G30A:C40U (ASL30) and A31G:U39C substitutions (ASL31) in *Ec*tRNA$^{Leu}_{CAA}$ (**Fig. 2B**). The efficiency of methylation of the 2 mutants by TrmL was similar to WT *Ec*tRNA$^{Leu}_{CAA}$ (**Fig. 3B**) and the steady-state kinetic constants were similar to the native enzyme parameters suggesting that the 2 conserved base pairs in the tRNA anticodon stem are not important for *Ec*TrmL recognition.

Essential recognition residues within the anticodon loop

Except for the wobble nucleotide that is the target of TrmL methylation, the other nucleotides of the anticodon loop are strictly conserved in *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$ (**Fig. 2A**). Ten mutations were performed in *Ec*tRNA$^{Leu}_{CAA}$. Three mutations at position 32, resulting in *Ec*tRNA$^{Leu}_{CAA}$-U32C/A/G (**Fig. 2B**), were overexpressed in the *E. coli* JW3581-1 strain. The methylation assays of dTrmL-tRNA$^{Leu}_{CAA}$-U32C/A showed results similar to those for dTrmL-tRNA$^{Leu}_{CAA}$, whereas dTrmL-tRNA$^{Leu}_{CAA}$-U32G was a poor substrate for *Ec*TrmL (**Fig. 3C**). The apparent K_m values and k_{cat} values for *Ec*tRNA$^{Leu}_{CAA}$-U32C and *Ec*tRNA$^{Leu}_{CAA}$-U32A were similar to those for WT *Ec*tRNA$^{Leu}_{CAA}$ (**Table 1**). These results suggest that residue U32 is not crucial for *Ec*TrmL recognition. U33 is at the position adjacent to the *Ec*TrmL catalytic site, so we performed a *Ec*tRNA$^{Leu}_{CAA}$-U33A mutation (**Fig. 2B**) and overexpressed this tRNA in *E. coli* JW3581-1. The efficiency of methylation of dTrmL-tRNA$^{Leu}_{CAA}$-U33A by TrmL was similar to that of WT *Ec*tRNA$^{Leu}_{CAA}$ (**Fig. 3D**). The apparent K_m value of *Ec*TrmL for *Ec*tRNA$^{Leu}_{CAA}$-U33A (4.46 μM) is approximately the same as for WT *Ec*tRNA$^{Leu}_{CAA}$ (4.12 μM) (**Table 1**), and the apparent k_{cat} values are also similar (0.40 s^{-1} and 0.55 s^{-1}, respectively) (**Table 1**). These results suggest that residue U33 is not a crucial residue for *Ec*TrmL recognition either. A35 is the other residue adjacent to the wobble base position, and previous work showed that TrmL catalyzed modification was significantly reduced on introduction of an *Ec*tRNA$^{Leu}_{CAA}$-A35U mutation into the test system *in vivo*.[16] Therefore, 3 mutations were performed at position 35, resulting in *Ec*tRNA$^{Leu}_{CAA}$-A35U/G/C (**Fig. 2B**). The total levels of isopentenylation by *Ec*MiaA *in vitro* for transcribed tRNA$^{Leu}_{CAA}$-A35U, -A35C and -A35G are the same (**Fig. S1**). Only *Ec*tRNA$^{Leu}_{CAA}$-A35U could be overexpressed in *E. coli* strain JW3581-1 (dTrmL-tRNA$^{Leu}_{CAA}$-A35U). *Ec*tRNA$^{Leu}_{CAA}$-A35G and -A35C were transcribed by T7 RNA polymerase and modified by *Ec*MiaA at position 37 *in vitro* (i^6A-Ts-tRNA$^{Leu}_{CAA}$-A35G/C). None of these 3 mutants could be recognized by TrmL, suggesting that A35 is the key residue for *Ec*TrmL recognition (**Fig. 3E**). Previous work showed that the A36-A37-A38 motif in the ASL of tRNA

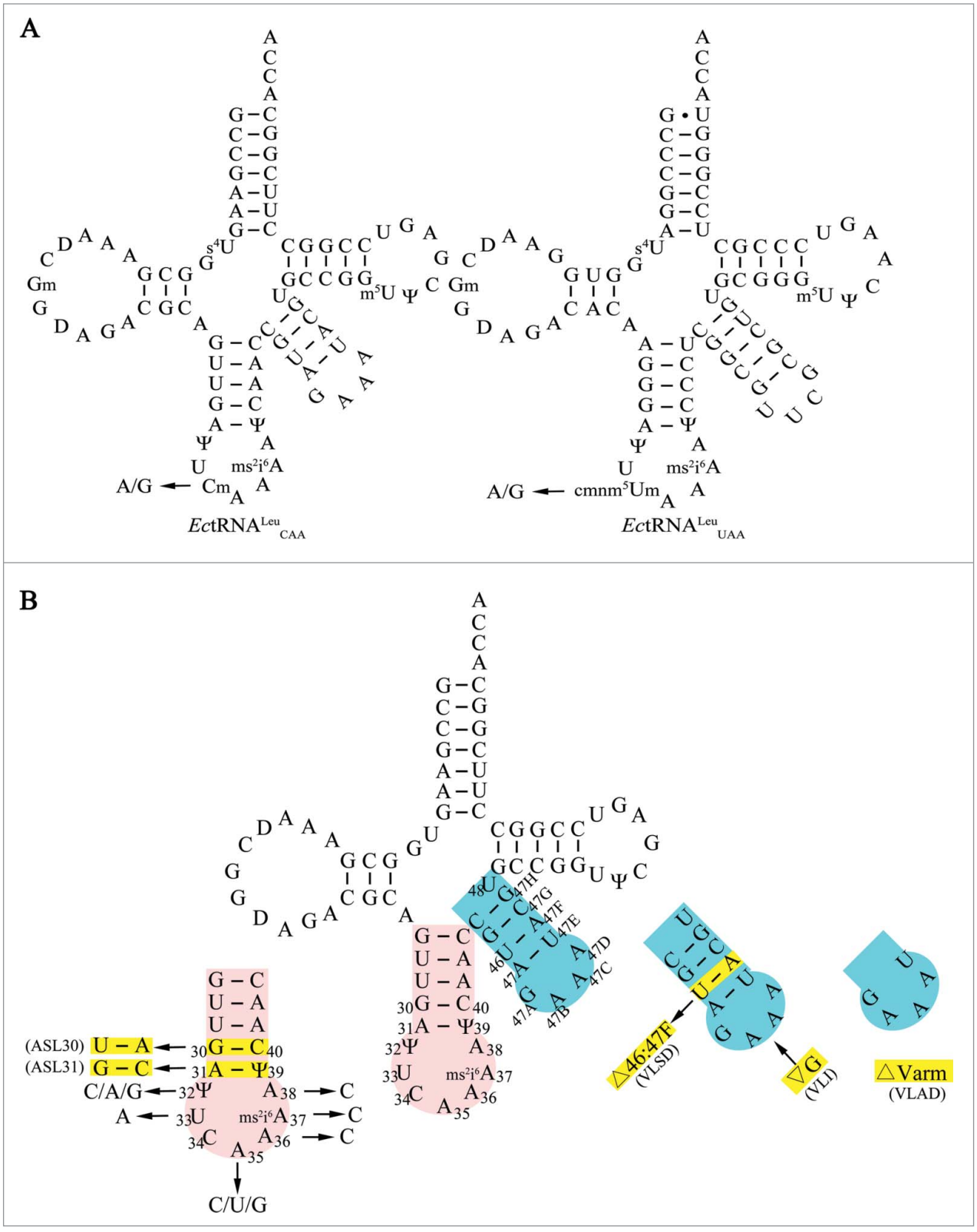

Figure 2. Overview of the *Ec*tRNA$^{Leu}_{CAA}$ mutations examined in this study. (**A**) Secondary structures of *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$. (**B**) Cloverleaf structure summarizing the *Ec*tRNA$^{Leu}_{CAA}$ mutations. The variants were created using site-directed mutagenesis. Cyan shows the variable region, pink shows the anticodon region, and bright yellow highlights the mutations. The arrows identify the locations of mutations; ψ, pseudouridine; D, dihydrouridine; Cm, 2′-O-methylcytidine; Um, 2′-O-methyluridine;ms^{2}i^{6}A, 2-methylthio-N^6-isopentenyladenosine; –, hydrogen bond between the standard bases; ·, hydrogen bond between the wobble base; △, deletion; ▽, insertion.

was strictly recognized by *Ec*MiaA0[40] so we checked whether these 3 residues are also recognized by *Ec*TrmL. Mutations at positions 37 and 38 resulting in *Ec*tRNA-$^{Leu}_{CAA}$-A37C and -A38C (**Fig. 2B**), could be overexpressed in *E. coli* JW3581-1 (dTrmL-tRNA$^{Leu}_{CAA}$-A37C and -A38C), but neither of these 2 mutants could be methylated by *Ec*TrmL (**Fig. 3F**). A mutant at position 36, resulting in *Ec*tRNA$^{Leu}_{CAA}$-A36C (**Fig. 2B**), however, could not be overexpressed in *E. coli* JW3581-1, and a DMAPP transfer assay showed that transcribed *Ec*tRNA$^{Leu}_{CAA}$-A36C could not be recognized by *Ec*MiaA *in vitro*. Therefore, we could not gain i^{6}A-Ts-tRNA$^{Leu}_{CAA}$-A36C for further methyl transfer assay.

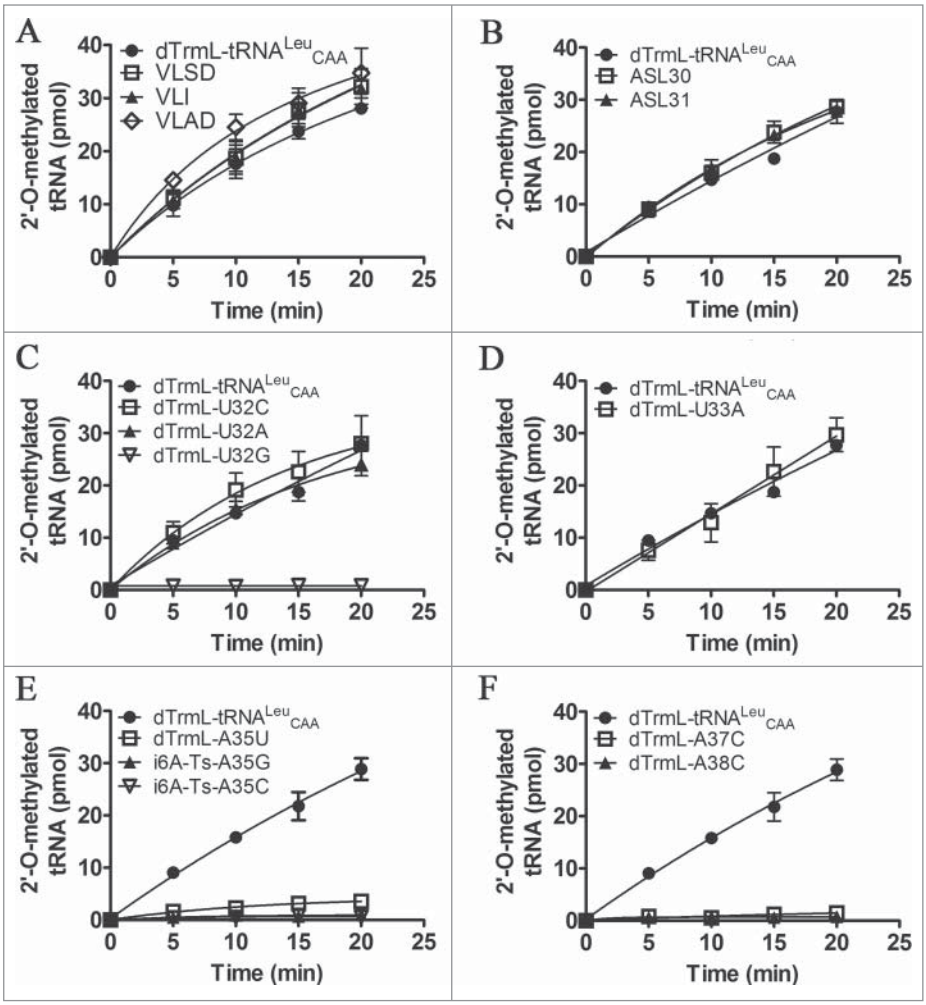

Figure 3. Essential recognition residues within the variable loop and anticodon stem loop of tRNA. (**A**) The capacity of *Ec*tRNA$^{Leu}_{CAA}$ with mutations in the variable loop to be methylated by *Ec*TrmL. VLSD, deletion of the U-A base pair at position 46-47F in the variable arm; VLI, a guanine nucleotide insertion into the middle of variable loop between positions 47B and 47C; VLAD, deletion of the whole variable arm. (**B**) The capacity of *Ec*tRNA$^{Leu}_{CAA}$ with mutations in the anticodon stem to be methylated by *Ec*TrmL. ASL30, substitution of the conserved G-C base pair at position 30-40 to A-U in the anticodon stem; ASL31, substitution of A-U at position 31-39 to G-C in the anticodon stem. (**C-F**) The capacity of *Ec*tRNA$^{Leu}_{CAA}$ with mutations in the anticodon loop to be methylated by *Ec*TrmL. All mutants were performed based on the sequence of *Ec*tRNA$^{Leu}_{CAA}$. dTrmL-U32C/A/G, -U33A, -A35U, -A37C and -A38C represent mutations at relevant positions overexpressed from a TrmL gene knockout strain. i^{6}A-Ts-A35G/C represent mutations at position 35 which were made by *in vitro* transcription, and followed with i^{6}A37 modification by MiaA *in vitro*. The negative control of the capacity of transcribed Ts-*Ec*tRNA$^{Leu}_{CAA}$ to be methylated by *Ec*TrmL was not shown in order to make the panels clearer. The results are the average of 3 independent experiments with standard deviations indicated.

Alteration of the main tRNA body does not affect recognition by *Ec*TrmL

*Ec*tRNA$^{Phe}_{GAA}$ belongs to the class I tRNAs, which have a short variable loop of 4-5 nucleotides. [41] Sequence alignment of *Ec*tRNA$^{Phe}_{GAA}$, *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$ showed that the sequences in the anticodon loop were almost identical between the 3 tRNAs, with the only difference at the wobble position, which is G, C, and U in *Ec*tRNA$^{Phe}_{GAA}$, *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$, respectively. However, the sequences of the 3 anticodon stems are completely different. The main tRNA body, including the amino acid acceptor stem, the variable loop region, the D loop region and the TψC loop region of *Ec*tRNA$^{Phe}_{GAA}$ also differs from that of *Ec*tRNA$^{Leu}_{CAA}$ or *Ec*tRNA$^{Leu}_{UAA}$. *Ec*tRNA$^{Phe}_{GAA}$ has a conserved A36-A37-A38 motif and possesses an i^{6}A modification introduced by MiaA at position 37. [40] Accordingly, we mutated G34 of *Ec*tRNA-$^{Phe}_{GAA}$ to C or U in order to mimic the anticodon loops of *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*RNA$^{Leu}_{UAA}$, respectively (**Fig. 4A**). Methylation assays of *Ec*tRNA$^{Phe}_{GAA}$-G34C and *Ec*tRNA$^{Phe}_{GAA}$-G34U showed results similar to those for *Ec*tRNA$^{Leu}_{CAA}$. These findings indicate that the sequences of the anticodon stem and variable loop are not essential for TrmL recognition (**Fig. 4B**). In the methylation assay, the apparent K_m value for *Ec*tRNA$^{Phe}_{GAA}$-G34C was 1.82 μM and the apparent k_{cat} was 0.58 s^{-1}; for *Ec*tRNA$^{Phe}_{GAA}$-G34U the values were 2.26 μM and 0.50 s^{-1}, respectively. These values are similar to those of WT *Ec*tRNA$^{Leu}_{CAA}$. Sequence alignment of *Ec*tRNA-$^{Ser}_{CGA}$ and *Ec*tRNA$^{Leu}_{CAA}$ showed that positions 32 and 35 of the anticodon loop were different with each other, while other positions including 33, 34, 36, 37 and 38 were the same. And the main tRNA body, including the amino acid acceptor stem, the variable loop region, the D loop region and the TψC loop region of *Ec*tRNA-$^{Ser}_{CGA}$ also differs from that of *Ec*tRNA$^{Leu}_{CAA}$ (**Fig. 4C**). We mutated G35 of *Ec*tRNA$^{Ser}_{CGA}$ to A in order to mimic the anticodon loop of *Ec*tRNA$^{Leu}_{CAA}$ (**Fig. 4C**). The total levels of isopentenylation of the plateau by *Ec*MiaA for transcribed tRNA$^{Ser}_{CGA}$ and tRNA$^{Ser}_{CGA}$-G35A are the same (**Fig. S2**). Further methylation assay showed that i^{6}A37 modified *Ec*tRNA$^{Ser}_{CGA}$-G35A but not

Table 1. Kinetic parameters of *Ec*TrmL methylation of various tRNA substrates

tRNAs	apparent K_m (μM)	apparent k_{cat} (min^{-1})	k_{cat}/K_m
$tRNA^{Leu}_{CAA}$[a]	3.39 ± 0.33	0.44 ± 0.07	0.13
$tRNA^{Leu}_{CAA}$-C34A	ND	ND	ND
$tRNA^{Leu}_{CAA}$-C34G	ND	ND	ND
$tRNA^{Leu}_{UAA}$-U34A	ND	ND	ND
$tRNA^{Leu}_{UAA}$-U34G	ND	ND	ND
$tRNA^{Leu}_{CAA}$ -VLI	3.12 ± 0.30	0.71 ± 0.10	0.23
$tRNA^{Leu}_{CAA}$ -VLSD	2.74 ± 0.39	0.75 ± 0.04	0.27
$tRNA^{Leu}_{CAA}$ -VLAD	3.35 ± 0.31	0.63 ± 0.09	0.19
$tRNA^{Leu}_{CAA}$ -ASL30	4.07 ± 0.26	0.85 ± 0.11	0.21
$tRNA^{Leu}_{CAA}$ -ASL31	3.39 ± 0.18	1.18 ± 0.02	0.35
$tRNA^{Leu}_{CAA}$ -U32C	3.54±0.19	0.99±0.14	0.28
$tRNA^{Leu}_{CAA}$ -U32A	5.37±0.27	1.20±0.16	0.22
$tRNA^{Leu}_{CAA}$ -U32G	ND	ND	ND
$tRNA^{Leu}_{CAA}$ -U33A	4.46 ± 0.26	0.40 ± 0.05	0.09
$tRNA^{Leu}_{CAA}$ -A35U	ND	ND	ND
$tRNA^{Leu}_{CAA}$ -A37C	ND	ND	ND
$tRNA^{Leu}_{CAA}$ -A38C	ND	ND	ND
$tRNA^{Phe}_{GAA}$-G34C	1.82 ± 0.22	0.58 ± 0.05	0.32
$tRNA^{Phe}_{GAA}$-G34U	2.26 ± 0.15	0.50 ± 0.12	0.22

[a]Values from Liu et al.[22]
The results are the average of three independent experiments with standard deviations indicated.

*Ec*tRNA$^{Ser}_{CGA}$, was the substrate of *Ec*TrmL (**Fig. 4D**). Taken together, these results suggest that the identity of anticodon loop only, but no other parts of the tRNA, is crucial for tRNA recognition by *Ec*TrmL.

Anticodon stem minihelices are substrates of *Ec*TrmL

In general, tRNA MTases can be divided into 2 groups based on their sensitivity to structural elements in the tRNA mole-cule. [42] The first group can only modify nucleosides using well-folded, full-length tRNA molecules as substrates, while the second group can efficiently modify truncated tRNA fragments.[42] We examined whether the L-shaped structure of tRNA or the anticodon stem loop alone was sufficient for *Ec*TrmL recognition. We constructed several *Ec*tRNA minisubstrates derived from *Ec*tRNA$^{Leu}_{CAA}$ (**Fig. 5A**). The minisubstrates were synthesized by *in vitro* transcription; they all lacked tRNA specific domains including the D-loop, the variable loop and the TψC regions, and only retained the tRNA anticodon stem-loop domain (**Fig. 5A**). For example, ASL-5 represents the tRNA anticodon stem-loop domain with 5 base pairs in the stem, and ASL-7 retained the 5 base pairs of the tRNA anticodon stem directly fused to 2 base pairs of the acceptor stem, and so on. The Acc-ASL mutant retained the anticodon stem-loop fused to the entire acceptor stem with a few nucleotides linking the 2 domains, which originally existed in the D-loop region and the TψC region. All the transcripts were modified by recombinant *Ec*MiaA at position 37 before they were tested as substrates in the tRNA methyl transfer assay. The effective concentrations of these transcribed minisubstrates or minihelices were determined by tRNA isopentenylation assay through detection of ^3H labeled DMAPP to ensure that these i^6A37 modified tRNA minihelices were used in the same effective concentration in later methyl transfer assays (see Materials and Methods).

The methylation capacities of the i^6A dimethylallylated minisubstrates revealed that i^6A-ASL-5 was a poor substrate, but the addition of extra base pairs in i^6A-ASL-7, i^6A-ASL-9, i^6A-ASL-11 and i^6A-Acc-ASL improved the methylation levels (**Fig. 5B,C**); these minihelices were methylated to levels comparable to i^6A modified *Ec*tRNA$^{Leu}_{CAA}$ transcript. Together, these results showed that an anticodon stem-loop minihelix extension of 2 base pairs is the minimal substrate requirement for *Ec*TrmL methylation.

Only pyrimidines at the wobble position are recognized by *Ec*TrmL

*Ec*TrmL could independently catalyze 2′-O-methylation at C34 and U34 in the *Ec*tRNA^{Leu} isoacceptors. Previous

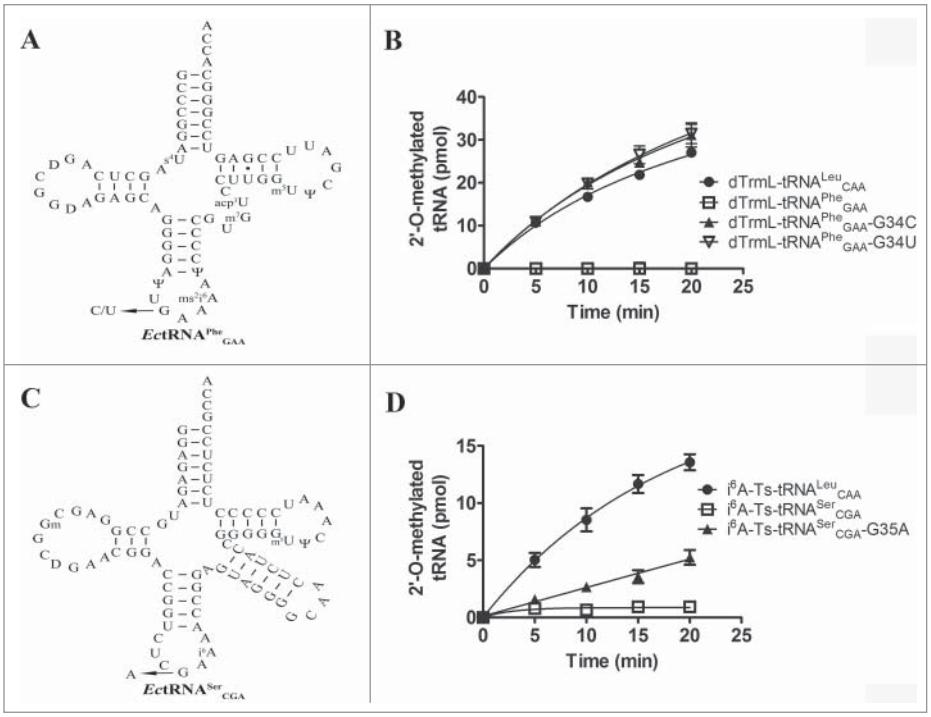

Figure 4. Alteration of the main tRNA body does not affect recognition by *Ec*TrmL. (**A**) Secondary structure of *Ec*tRNA$^{Phe}_{GAA}$ highlighting the mutations at position 34 (*Ec*tRNA$^{Phe}_{GAA}$-G34C and *Ec*tRNA$^{Phe}_{GAA}$-G34U). (**B**) The capacity of dTrmL-tRNA$^{Phe}_{GAA}$-G34C and dTrmL-tRNA$^{Phe}_{GAA}$-G34U to be methylated by *Ec*TrmL. (**C**) Secondary structure of *Ec*tRNA$^{Ser}_{CGA}$ highlighting the mutation at position 35 (*Ec*tRNA$^{Ser}_{CGA}$-G35A). (**D**) The capacity of i^6A-Ts-tRNA$^{Ser}_{CGA}$ and i^6A-Ts-tRNA$^{Ser}_{CGA}$-G35A to be methylated by *Ec*TrmL. The results are the average of 3 independent experiments with standard deviations indicated.

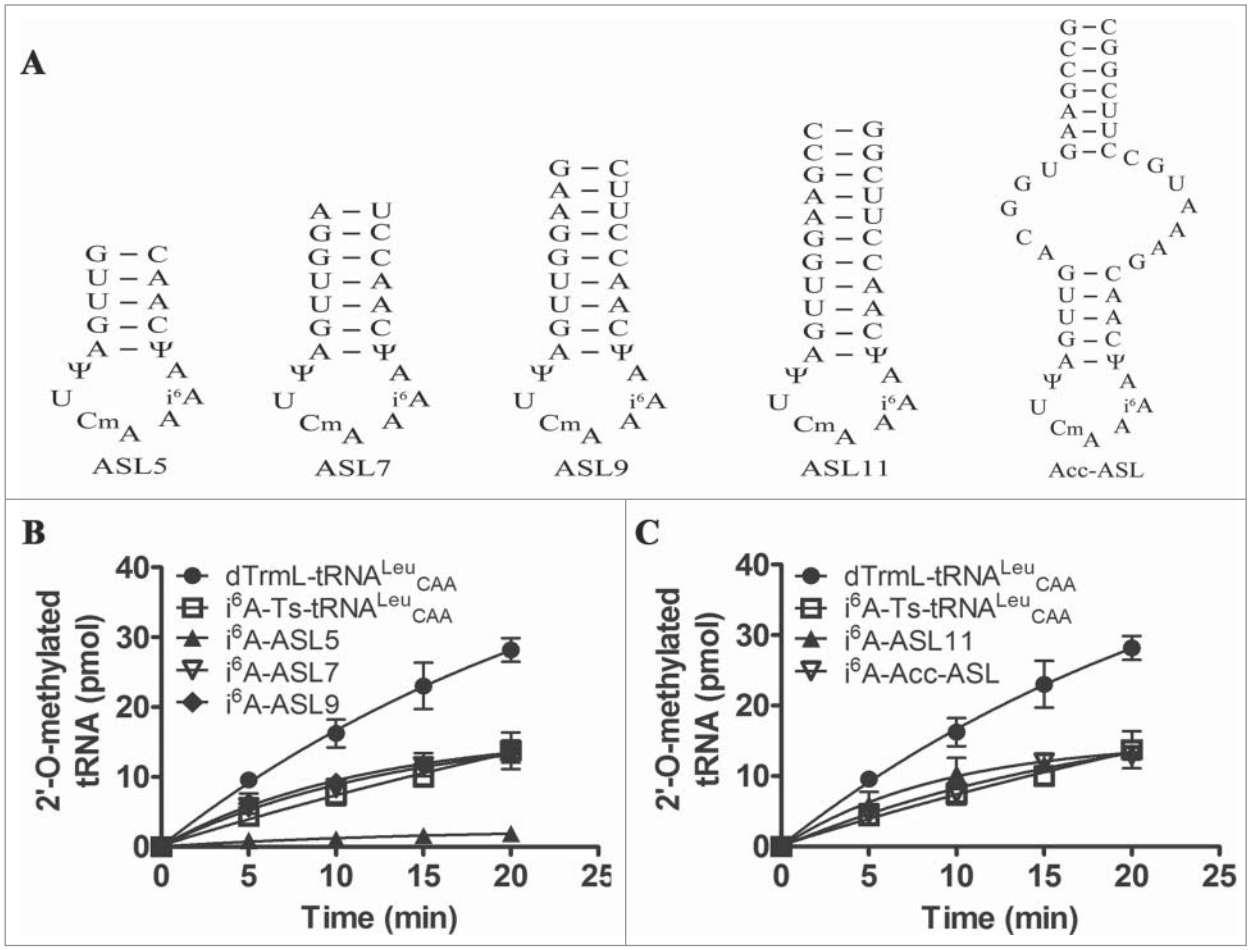

Figure 5. Truncated tRNA minihelices could be recognized by *Ec*TrmL. (**A**) Secondary structure of the minihelices of the ASL of *Ec*tRNA$^{Leu}_{CAA}$ (ASL5/7/9/11 and Acc-ASL). (**B**) and (**C**) show the capacity of the minihelices to be methylated by *Ec*TrmL. The results are the average of 3 independent experiments with standard deviations indicated.

in vivo study showed that TrmL could not methylate tRNA$^{Leu}_{CAA}$-C34A in *E. coli*.[16] To determine whether *Ec*TrmL could recognize a purine nucleotide at position 34, we mutated C34 or U34 in the tRNA^{Leu} isoacceptors to A34 or G34, resulting in *Ec*tRNA$^{Leu}_{CAA}$-C34A/G or *Ec*tRNA$^{Leu}_{UAA}$-U34A/G, respectively (**Fig. 2A**). Mutants C34A and U34A were purified following *in vivo* overexpression as described above, however, mutants C34G and U34G could not be overexpressed and were transcribed by T7 RNA polymerase before being modified by *Ec*MiaA at position 37 *in vitro*. The methyl transfer assay by recombinant *Ec*TrmL showed no detectable activity when using *Ec*tRNA$^{Leu}_{CAA}$ or *Ec*tRNA$^{Leu}_{UAA}$ with purine substitutions at position 34, whether the i^{6}A37 modification was present or not (**Fig. 6A,B**). This finding shows that only pyrimidine nucleotides at position 34 are substrates of *Ec*TrmL.

Discussion

The i^{6}A37 modification has been found to be widespread and conserved. [43] Defects in this modification were recently found in human pathogenic mutations.[44] In bacteria, the ms^{2}i^{6}A37 modification is carried out in 2 successive steps by MiaA and MiaB.[45] It has been shown that the ms^{2}i^{6}A37 modification occurs earlier than other modifications in the anticodon stem-loop and a tRNA helical stem-loop containing an A36-A37-A38 motif are determinants for MiaA recognition.[40] In *E. coli*, both tRNA$^{Leu}_{CAA}$ and tRNA$^{Leu}_{UAA}$ isoacceptors contain the ms^{2}i^{6}A37 modification. Interestingly, *in vitro* transcribed tRNA$^{Leu}_{CAA}$ and tRNA$^{Leu}_{UAA}$ without modifications are not substrates of TrmL methylase, suggesting a sequential process starting with the ms^{2}i^{6}A37 modification. [20] We show here that synthetic transcripts of tRNA$^{Leu}_{CAA}$ and tRNA$^{Leu}_{UAA}$ with the first i^{6}A37 modification, catalyzed by recombinant MiaA, are substrates of TrmL. Similarly, tRNA^{Leu} purified from an *E. coli* MiaA-knockout strain was an effective substrate of TrmL after *in vitro* introduction of i^{6}A37 modification by recombinant MiaA. These data demonstrate that only the i^{6}A37 modification is a strict prerequisite for TrmL-catalyzed methylation and these results are consistent with a previous study showing that Yibk (TrmL) is inactive with tRNA substrates deprived of the fully modified ms^{2}i^{6}A37 base.[16] Altogether, the results confirm that the modifications of

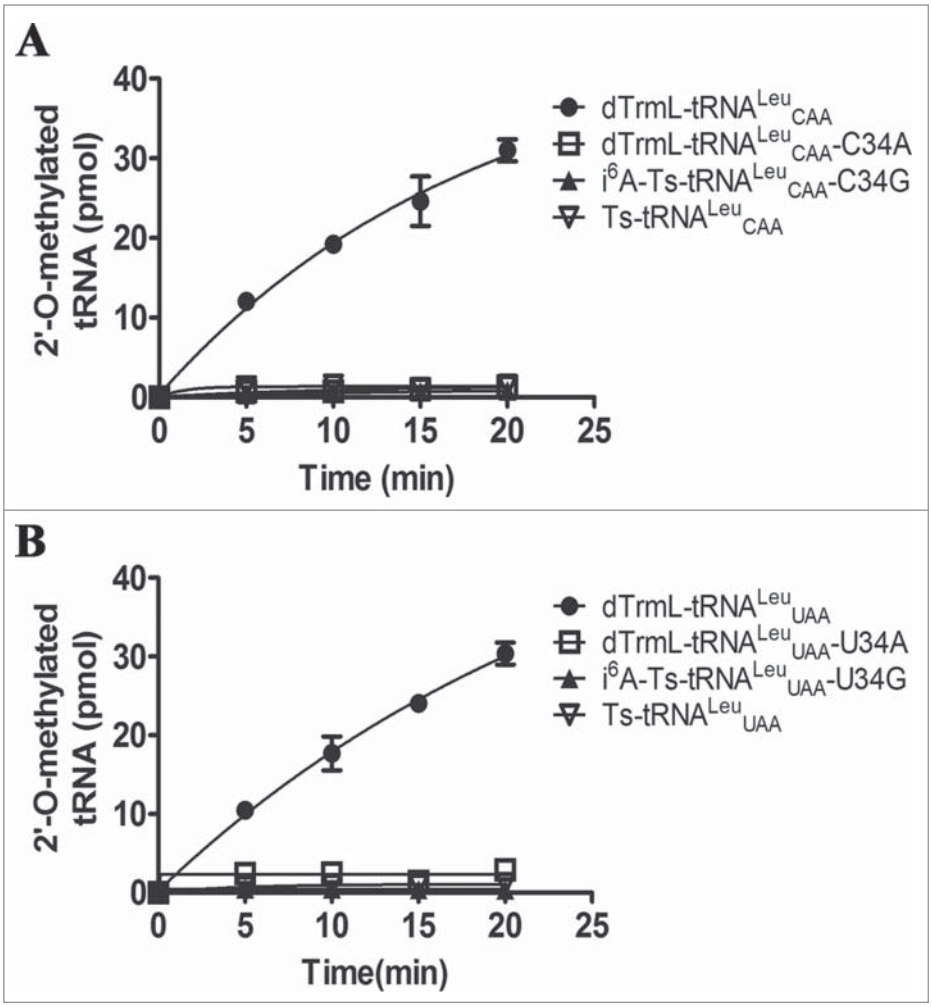

Figure 6. Only pyrimidines at the wobble position are recognized by TrmL. (**A**) and (**B**) The capacity of dTrmL-*Ec*tRNA$^{Leu}_{CAA}$-C34A, dTrmL-*Ec*tRNA$^{Leu}_{UAA}$-U34A, i^{6}A-Ts-*Ec*tRNA$^{Leu}_{CAA}$-C34G and i^{6}A-Ts-*Ec*tRNA$^{Leu}_{UAA}$-U34G to be methylated by *Ec*TrmL. dTrmL-*Ec*tRNA$^{Leu}_{CAA}$-C34A and dTrmL-*Ec*tRNA$^{Leu}_{UAA}$-U34A were overexpressed from a TrmL gene knockout strain, i^{6}A-Ts-*Ec*tRNA$^{Leu}_{CAA}$-C34G and i^{6}A-Ts-*Ec*tRNA$^{Leu}_{UAA}$-U34G were made by *in vitro* transcription by T7 RNA polymerase followed with i^{6}A37 modification by MiaA *in vitro*. The results are the average of 3 independent experiments with standard deviations indicated.

the wobble base and the purine at position 37 occur in chronological order *in vivo*.

tRNA$^{Leu}_{CAA}$ and tRNA$^{Leu}_{UAA}$ are the only 2 RNA substrates of TrmL. The mechanism by which TrmL distinguishes its targets from all other tRNAs, which all have similar L-shaped tertiary structure, is unclear. Our data indicated that only pyrimidine nucleotides at the wobble position could be methylated by TrmL, which is consistent with previous results showing that TrmL could not methylate *Ec*tRNA$^{Leu}_{CAA}$-C34A.[16] *Ec*tRNA$^{Phe}_{GAA}$ is a type I tRNA and naturally does not contain a 2′-O-methylation modification at position 34. However, with the exception of position 34, which is a G, the 6 other nucleotides of the anticodon loop of *Ec*tRNA$^{Phe}_{GAA}$ are identical to *Ec*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$, although the remaining components of the tRNA body are different. Surprisingly, *Ec*tRNA$^{Phe}_{GAA}$-G34C and *Ec*tRNA$^{Phe}_{GAA}$-G34U mutants were methylated by *Ec*TrmL as efficiently as *Ec*tRNA$^{Leu}_{CAA}$.

Our results also showed that TrmL could accommodate many changes in the tRNA$^{Leu}_{CAA}$ structure. For instance, changes in the variable loop of *Ec*tRNA$^{Leu}_{CAA}$ do not affect TrmL activity. We further showed that several residues of the anticodon loop, but not the anticodon stem, were crucial for TrmL activity. Mutations of U32 and U33 preserved *Ec*TrmL methylation activity. The nucleotide at position 32 in *E. coli* tRNAs is generally a semi-conserved cytidine or uridine residue,[46] and U33 is invariant in all tRNAs.[40,47] Therefore, that TrmL does not use these conserved nucleotides at positions 32 and 33 as recognition determinants seems reasonable. A35 functions as an identity element for *Ec*TrmL recognition, which is consistent with the results that TrmL could not methylate tRNA$^{Leu}_{CAA}$-A35U in *E. coli*.[16] The tRNA mutant A36C could not be overexpressed in *E. coli* JW3581-1 *in vivo*, neither its transcript could be recognized by *Ec*MiaA *in vitro*. The tRNA mutants A37C and A38C resulted in loss of the i^{6}A37 modification and therefore of recognition by TrmL. Because A36-A37-A38 motif is strictly recognized by MiaA, any change of it could result in loss of the i^{6}A37 modification. Therefore, the reason for these mutants could not be recognized by TrmL may be the loss of i^{6}A37 modification. However, it is also probable that some nucleotide(s) in the A36-A37-A38 motif is/are the recognition determinant(s) for TrmL through direct interactions. Mutant *Ec*tRNA$^{Ser}_{CGA}$-G35A could not be overexpressed in the TrmL gene deletion strain. So we transcribed it by T7 RNA polymerase and modified by *Ec*MiaA at position 37 *in vitro*. Our results show that *Ec*tRNA$^{Ser}_{CGA}$-G35A turns *Ec*tRNA$^{Ser}_{CGA}$ into a substrate for TrmL. Although this mutant could be recognized by TrmL, the efficiency of *Ec*TrmL to methylate *Ec*tRNA$^{Ser}_{CGA}$-G35A is rather low compared with i^{6}A37 modified *Ec*tRNA$^{Leu}_{CAA}$ transcripts. Because main body of tRNA, including anticodon stem, amino acid acceptor stem, the variable loop region, the D loop region and the TψC loop region of *Ec*tRNA$^{Ser}_{CGA}$ differ from that of *Ec*tRNA$^{Leu}_{CAA}$, the reason for *Ec*tRNA$^{Ser}_{CGA}$-G35A being a relatively poor substrate of TrmL might be due to slightly local conformation change and/or some steric hindrance. Taken together, these results suggest that the tRNA anticodon loop, but not any other

part of the tRNA, as well as the i^6A37 modification, are crucial for TrmL recognition. In a last assay to further test the critical role of the anticodon loop of tRNA, we showed that *Ec*TrmL could modify tRNA minihelices exhibiting the anticodon stem-loop structure (ASL) and the i^6A37 modification catalyzed by *Ec*MiaA.

In summary, these results suggest that *Ec*TrmL recognizes its substrate tRNAs based on (i) the stem-loop structure of ASL extended by 2 base pairs, (ii) the correct identity elements of the anticodon loop, (iii) a pyrimidine at the wobble position, and (iv) a preexisting i^6A37 modification (**Fig.** 7). Thus, it seems that TrmL may stretch out many hands and arms, to identify those recognition determinants at the same time (**Fig.** 7). This recognition pattern is very different from the other 3 SPOUT tRNA MTases (TrmH, TrmD and TrmJ) in prokaryotes. TrmH is one of the methyltransferases which catalyzes 2′-O-methylation at position 18 of tRNA,[21-24] and TrmH exhibits different recognition of its tRNA substrates than TrmL. TrmHs can be divided into 2 subclasses; one can recognize all tRNA species, whereas the other, including *Ec*TrmH, can only modify a subset of tRNA species.[27] Early study suggested that G18G19 and the D-arm structure of the tRNA are essential requirements for recognition by *Thermus thermophilus* TrmH, [21] but further work showed that *T. thermophilus* TrmH recognized G18 with some flexibility and the oxygen 6 of G18 was the crucial determinant for TrmH recognition.[25] TrmD is a tRNA (m^1G37) MTase.[28-32] It belongs to the first group of tRNA modifying enzymes, which don't need the L-shaped structure of tRNA for substrate recognition.[42] TrmD can recognize and methylate short RNA structures such as the stem-loop structure of ASL, similar behavior to TrmL.[28,31,33,36,48] G36-G37 is the known recognition determinant for TrmD.[34,35,37] *Ec*TrmJ catalyzes 2′-O-methylation at position 32 of tRNA.[38] *Ec*TrmJ needs the full-length tRNA and correct identity elements within the D stem and loop region, which is totally different from TrmL.[39]

Our data showed that 2′-O-methylation at the wobble position by TrmL requires a previous i^6A37 modification. It is, however, unclear how the i^6A37 modification influences tRNA recognition by TrmL. A direct interaction could occur between TrmL and the bulky isopentenyl group added by MiaA at A37, which could act as a recognition determinant. Alternatively, the isopentenyl group could change the structure of the anticodon loop region, which would affect the interaction with TrmL. Indeed, it was reported that the i^6A37 modification changes the structural conformation of *E. coli* $tRNA^{Phe}_{GAA}$ ASL, as observed in NMR structures.[49] The unmodified ASL molecule adopts a stem-loop conformation composed of 7 base-pairs and a compact 3 nucleotide loop, which is obviously different from the classical loop observed in fully modified tRNA which contains a U-turn motif at position 33.[49] Therefore, the conformational change of ASL resulting from the i^6A37 modification might also be important for recognition by TrmL.

Methylation is one of the most common and ubiquitous RNA modifications, and it is present in many different types of cellular RNAs, including rRNA, mRNA, tRNA, microRNA and other small RNAs. Recently, RNA demethylases were identified that

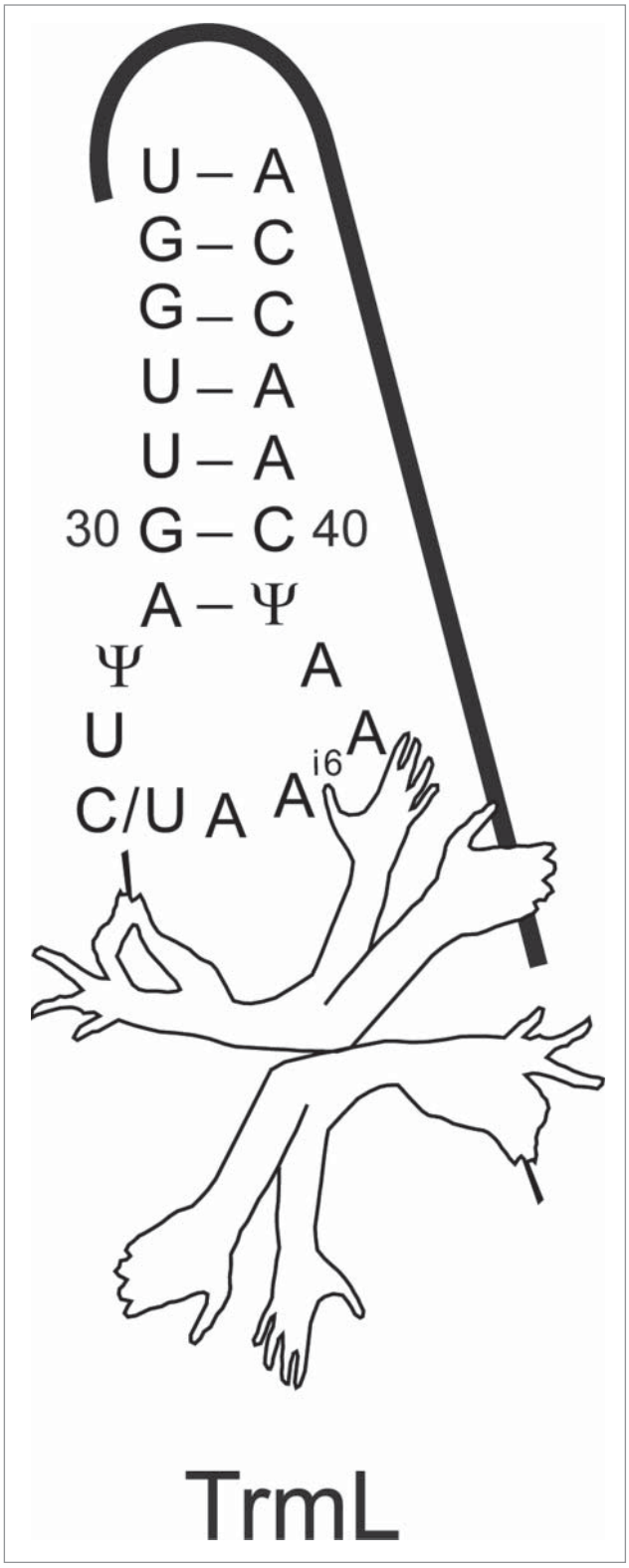

Figure 7. A scheme to summarize the determinant elements of tRNA recognized by TrmL. Hands and arms show that TrmL is recognizing the ASL including the stem-loop structure, the correct identity elements, a pyrimidine at the wobble position and a preexisting i^6A37 modification.

were found to be involved in fundamental regulatory roles in many important life processes, including germline development, cellular signaling, and circadian rhythm control.[50,51] Intriguing questions regarding how these RNA MTases or demethylases recognize or bind to their RNA substrates remain, since unlike DNA binding domains that share common structural motifs like zinc fingers, leucine zippers etc., little is known about RNA binding motifs. Our study of how the SPOUT MTase precisely recognizes its RNA substrates sheds light on RNA substrate recognition.

Material and Methods

Materials

[Methyl-^{3}H] SAM (78.0 Ci/mmol) was purchased from PerkinElmer Inc. (Waltham, MA, USA); [^{3}H] DMAPP (20.0 Ci/mmol) was obtained from BIOTREND Chemicals (USA); SAM was purchased from NEW ENGLAND Biolabs Inc..; Dimethylallyl diphosphate (DMAPP), dithiothreitol (DTT), NTPs, 5′-GMP, pyrophosphate, Tris-base, β-mercaptoethanol (β-Me), $MgCl_2$, NaCl and KCl were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA); Isopropyl β-D-thiogalactoside (IPTG) was obtained from AMRESCO (OH, USA); Nitrocellulose membranes (0.22 μm) were purchased from Merck Millipore (Darmstadt, Germany); Primers for PCR were synthesized by BioSune (Shanghai, China); Nickel-nitrilotriacetic (Ni-NTA) Superflow was purchased from Qiagen Inc.. (Germany); KOD-plus mutagenesis kit, Pyrobest DNA polymerase and the dNTP mixture were obtained from Takara (Japan); T4 polynucleotide kinase, T4 DNA ligase, Ribonuclease inhibitor and all restriction endonucleases were obtained from Fermentas/Thermo Scientific (MA, USA); The *E. coli* JW3581-1 and JW4129-2, strains in which the gene of TrmL and MiaA were deleted respectively, were purchased from the *E. coli* genetic stock center (Yale University, New Haven, CT, USA); PrepHT 300SB-C18 was purchased from Agilent Technologies (CA, USA).

Preparation of tRNAs and mutagenesis

The genes encoding *Ec*tRNAs including *Ec*tRNA$^{Leu}_{CAA}$, *Ec*tRNA$^{Leu}_{UAA}$ and *Ec*tRNA$^{Phe}_{GAA}$ were amplified from the genome of *E. coli* MG1655. Site-directed mutagenesis of *Ec*tRNAs was performed using the KOD-plus mutagenesis kit as described previously. [52] The 2′-O-methylation deficient tRNAs were overexpressed in *E. coli* JW3581-1, and the isopentenylation deficient tRNA was overexpressed in *E. coli* JW4129-2. These *in vivo* overexpressed tRNAs were purified by DEAE-Sepharose chromatography, urea denaturing PAGE and C18 reversed-phase HPLC chromatography as described previously.[20] Unmodified tRNAs were made by *in vitro* transcription by T7 RNA polymerase according to previous protocols.[53] The tRNA minihelices were synthesized by *in vitro* transcription of DNA templates that were made by direct primer annealing.[31] The A37-dimethylallyl-modified (i^{6}A37) tRNAs were made using recombinant *E. coli* MiaA (*Ec*MiaA) and dimethylallyl diphosphate (DMAPP) in the tRNA isopentenylation assay. The tRNA concentration was determined by UV absorbance at 260 nm, and the molar absorption coefficient was calculated according to the sequence of each tRNA.

tRNA methyl transfer assay

The gene encoding *E. coli* TrmL (*Ec*TrmL) was inserted into vector pET30b and expressed in *E. coli* BL21 (DE3). The protein purification and the tRNA methylation assay were performed as described previously.[20] For measurement of methyl transfer capability, 7 μM wild-type, mutant *Ec*tRNA^{Leu} and i^{6}A37 modified tRNA minihelices were used as substrates. The reaction was performed under the same conditions, including 0.5 μM TrmL, as previously described.[20] At time intervals ranging between 5 and 20 min, aliquots of 10 μL were removed, absorbed on paper discs and precipitated in trichloroacetic acid. The kinetic parameters of the methylation reactions of *Ec*tRNAs were determined by using a range of 0.5–20 μM tRNA, 100 μM SAM and 0.2 μM *Ec*TrmL.

tRNA isopentenylation assay

The gene encoding *Ec*MiaA was inserted into the pET30b vector with an N-terminal His tag and expressed in *E. coli* BL21 (DE3). The protein was purified following a previously described protocol used for His-tagged *Ec*TrmL.[20] The isopentenylation reaction contained 50 mM Tris-HCl (pH 7.5), 3.5 mM $MgCl_2$, 5 mM DTT, 1 mg/mL BSA, 7 μM *Ec*tRNA, 50 μM DMAPP and 1 μM *Ec*MiaA. The reaction was incubated at 37°C for 20 min before quenching by phenol extraction. To measure the effective concentrations of transcribed minihelices, reactions were performed under identical conditions at 37°C, ^{3}H labeled DMAPP was used, and aliquots of 5 μL were removed, absorbed on paper discs and precipitated in trichloroacetic acid, at time intervals ranging from 5 to 60 min. After the precise effective concentration was confirmed, these transcribed minihelices were modified by *Ec*MiaA using non isotopic labeled DMAPP, in the same conditions as for the previous reaction, for 20 min, before quenching by phenol extraction.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out as previously described.[20] Then, 80 nM tRNA with a range of 0.75–10 μM *Ec*TrmL was incubated in a 30 μL reaction volume at 37°C for 20 min. After incubation, each sample was loaded onto a 6% polyacrylamide native gel immediately after adding loading buffer. The gel was stained with ethidium bromide.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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