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

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Abstract: 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 EcTrmL 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 (i6) at A37. In addition, TrmL only methylates pyrimidines but not purine residues at the wobble position, and the 2'-O-methylation relies on prior N9-isopentenyladenosine modification at position 37.

Keywords: recognition determinants, TrmL, tRNA modification, wobble base, 2'-O-methyltransferase

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 cm5U 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} (EcRNA_{Leu}^{CAA}) and tRNA_{Leu}^{UAA} (EcRNA_{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 TrmL¹⁶,²⁰ TrmL functions as a homodimer...
using basic amino acid surface residues of TrmL for tRNA recognition. 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. Substitution at G18G19 causes deficiencies in methyl transfer activity, and the oxygen 6 atom of G18 is a key recognition element for TrmH. TrmD and TrmJ act on the tRNA anticodon loop. TrmD is a tRNA (m1G37) MTase, and TrmJ is the tRNA Um32/Cm32 methyltransferase. TrmL requires only an anticodon stem-loop structure with an oxygen 6 atom of G18 is a key recognition element for TrmL. TrmD and TrmJ act on the tRNA substrates than TrmL. TrmH catalyzes the 2'-O-methylation of tRNA G18.  

**Results**

\( \text{i}^{\text{A37}} \) is sufficient to restore 2'-O-methylation at C34 of EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\) transcripts in vitro

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

We next analyzed the binding affinities of EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\) with EctTrmL using an electrophoretic mobility shift assay (Fig. 1C). Three tRNAs, including native EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\) extracted from strain JW3581-1 and EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\) transcript with or without the ms\(^{i}37\) modification, were incubated with increasing concentrations of EctTrmL (0.75–10 μM). For native EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\), the shift representing the EctTrmL-tRNA complex (tRNA bound 1) was initially observed at an enzyme concentration of 0.75 μM. A supershift was observed above 3.0 μM EctTrmL, representing a larger molecular mass complex or aggregate (tRNA bound 2). Transcripts of EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\) without any natural post-transcriptional modifications showed no detectable binding to EctTrmL in the range of enzyme concentration tested. The assay with \( \text{i}^{\text{A37}} \) modified EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\) transcripts showed a decreased binding affinity to EctTrmL when compared with native EctRNA\(_{\text{Leu}}\)\(_{\text{CAA}}\). The shift was first observed at 1.5 μM enzyme, and a supershift was
observed above 3.0 μM EcTrmL. This assay showed that although
the affinity of 3'A-Ts-tRNA^{Leu}_{CAA} to EcTrmL was lower than for
WT tRNA^{Leu}_{CAA}, the 3'A modification had a consequent effect
on the binding strength of tRNA transcripts for EcTrmL.

Construction of tRNA^{Leu}_{CAA} mutants

SPOT MTase TrmL functions as homodimer by using basic
surface amino acid residues to recognize its tRNA substrates.
Only tRNA^{Leu}_{CAA} and tRNA^{UAA}_{UUU} isoacceptors are substrates
for TrmL, however, nucleotides that determine the “TrmL iden-
tity” have not yet been determined. We designed a systematic
approach to replace nucleotides in tRNA^{Leu}_{CAA}. Figure 2A
shows the secondary structure of tRNA^{Leu}_{CAA} and tRNA^{Leu}_{UAA}.
The two anticodon loops are conserved except
for nucleotide 34, which is C in tRNA^{Leu}_{CAA} and U in tRNA-
NA^{Leu}_{UAA}. The anticodon stem and variable loop do not show
sequence conservation, especially within the variable loop region
where tRNA^{Leu}_{UAA} has a longer stem than tRNA^{Leu}_{CAA}.Based on these variations, we performed site-directed mutagenesis
in tRNA^{Leu}_{CAA} as follows: deletions within the whole vari-
able arm, which is consistent with most tRNAs with the exception of tRNA^{Leu}_{UAA} and tRNA^{Leu}_{UAA} (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 nucleo-
tide from positions 32 to 38 by changing purines to pyrimidines
or vice versa, resulting in tRNA^{Leu}_{CAA}-U32C/A/G, tRNA^{Leu}_{CAA}-U33A, tRNA^{Leu}_{CAA}-A35G/U/C, tRNA^{Leu}_{CAA}-A36C, tRNA^{Leu}_{CAA}-A37C, and tRNA^{Leu}_{CAA}-A38C
(Fig. 2B). Most of these tRNAs were overexpressed and purified
by phenol extraction followed by DEAE-Sepharose chromatogra-
phy 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 EmiA at position 37
in vitro. As a control, wild type tRNA^{Leu}_{CAA} was purified in
the TrmL gene deletion strain (dTmL-tRNA^{Leu}_{CAA}) or trans-
scribed by T7 RNA polymerase in vitro (Ts-tRNA^{Leu}_{CAA}).

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

tRNA^{Leu}_{UAA} belongs 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 EcTrmL approaches the wobble position of
EcRNA. To address this possibility, we examined the effect of
dracastic mutations in the variable arm and loop of 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 tRNA^{Leu}_{CAA} (dTmL-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 nucleo-
tide insertions (VLI) had no significant effect on the methylation
parameters, indicating that EcTrmL 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 EcTrmL

Sequence comparison of the 2 tRNA^{Leu}_{CAA} isoacceptors showed
that the fourth and fifth base pairs in the anticodon stem of
the 2 tRNA^{Leu}_{CAA} isoacceptors are conserved (Fig. 2A). We
performed G30A:C40U (ASL30) and A31G:U39C substitutions
(ASL31) in tRNA^{Leu}_{CAA} (Fig. 2B). The efficiency of methyla-
tion of the 2 mutants by TrmL was similar to WT 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 EcTrmL 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 tRNA^{Leu}_{CAA} and tRNA^{Leu}_{UAA} (Fig. 2A).
Ten mutations were performed in tRNA^{Leu}_{CAA}. Three muta-
tions at position 32, resulting in tRNA^{Leu}_{CAA}-U32C/A/G
(Fig. 2B), were overexpressed in the E. coli JW3581-1 strain. The
methylation assays of dTmL-tRNA^{UAA}-U32C/A showed
results similar to those for dTmL-tRNA^{Leu}_{CAA}, whereas dTmL-
tRNA^{UAA}-U32G was a poor substrate for EcTrmL (Fig. 3C).
The apparent K_m values and k_cat values for tRNA^{Leu}_{CAA}-U32C
and tRNA^{UAA}-U32A were similar to those for WT tRNA-
NA^{Leu}_{CAA} (Table 1). These results suggest that residue U32 is not
crucial for EcTrmL recognition. U33 is at the position adjacent to
the EcTrmL catalytic site, so we performed a tRNA^{Leu}_{CAA}-
U33A mutation (Fig. 2B) and overexpressed this tRNA in E. coli
JW3581-1. The efficiency of methylation of dTmL-tRNA^{Leu}_{CAA}-
U33A by TrmL was similar to that of WT tRNA^{Leu}_{CAA}
(Fig. 3D). The apparent K_m value of EcTrmL for tRNA^{Leu}_{CAA}-
U33A (4.46 μM) is approximately the same as for WT tRNA-
NA^{UAA}-U33A (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
EcTrmL 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 tRNA^{Leu}_{CAA}-A35U mutation into the test system in vitro. Therefore, 3 mutations were performed at position 35,
resulting in tRNA^{Leu}_{CAA}-A35U/G/C (Fig. 2B). The total levels
of isopentenylation by EmiA in vitro for transcribed tRNA^{Leu}_{CAA}-
A35U, -A35C and -A35G are the same (Fig. S1). Only tRNA-
NA^{Leu}_{CAA}-A35U could be overexpressed in E. coli strain JW3581-
1 (dTmL-tRNA^{Leu}_{CAA}-A35U). tRNA^{Leu}_{CAA}-A35G and -A35C were
transcribed by T7 RNA polymerase and modified by EmiA
at position 37 in vitro (3'A-Ts-tRNA^{Leu}_{CAA}-A35G/C). None of
these 3 mutants could be recognized by TrmL, suggesting that
A35 is the key residue for EcTrmL 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: $\psi$, pseudouridine; D, dihydrouridine; Cm, 2'-O-methylcytidine; Um, 2'-O-methyluridine; ms$^2$PA, 2-methylthio-N$^6$-isopentenyladenosine; $\cdot$, hydrogen bond between the standard bases; $\cdot\cdot$, hydrogen bond between the wobble base; $\triangle$, deletion; $\triangledown$, insertion.
was strictly recognized by EcMiaA so we checked whether these 3 residues are also recognized by EcTrmL. Mutations at positions 37 and 38 resulting in EcRNALeu\(^\text{CAA-A37C and -A38C}\) (Fig. 2B), could be overexpressed in \(E.\) coli JW3581-1 (dTTrmL-tRNALeu\(^\text{CAA-A37C and -A38C}\)), but neither of these 2 mutants could be methylated by EcTrmL (Fig. 3F). A mutant at position 36, resulting in EcRNA\(^{\text{L}}\)\text{C-A36C} (Fig. 2B), however, could not be overexpressed in \(E.\) coli JW3581-1, and a DMAPP transfer assay showed that transcribed EcRNA\(^{\text{L}}\)\text{C-A36C} could not be recognized by EcMiaA \text{in vitro}. Therefore, we could not gain i6A-Ts-tRNA\(^{\text{L}}\)\text{C-A36C} for further methyl transfer assay.

Alteration of the main tRNA body does not affect recognition by EcTrmL

EcRNA\(^{\text{Phe}}\)\text{GAA} belongs to the class I tRNAs, which have a short variable loop of 4-5 nucleotides. Sequence alignment of EcRNA\(^{\text{Phe}}\)\text{GAA}, EcRNA\(^{\text{Leu}}\)\text{CAA} and EcRNA\(^{\text{L}}\)\text{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 EcRNA\(^{\text{Phe}}\)\text{GAA}, EcRNA\(^{\text{Leu}}\)\text{CAA} and EcRNA\(^{\text{L}}\)\text{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 T\(\text{D}\)C loop region of EcRNA\(^{\text{Phe}}\)\text{GAA} also differs from that of EcRNA\(^{\text{Leu}}\)\text{CAA} or EcRNA\(^{\text{L}}\)\text{UAA}. EcRNA\(^{\text{L}}\)\text{Phe}\text{GAA} has a conserved A36-A37-A38 motif and possesses an i6Am modification introduced by MiaA at position 37. Accordingly, we mutated G34 of EcRNA\(^{\text{Phe}}\)\text{GAA} to C or U in order to mimic the anticodon loops of EcRNA\(^{\text{Leu}}\)\text{CAA} and EcRNA\(^{\text{L}}\)\text{UAA}, respectively (Fig. 4A). Methylation assays of EcRNA\(^{\text{Phe}}\)\text{GAA-G34C and -G34U} showed results similar to those for EcRNA\(^{\text{L}}\)\text{C-G34C and -G34U}. 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 EcRNA\(^{\text{Phe}}\)\text{GAA-G34C was 1.82 \(\mu\)M and the apparent \(k_{cat}\) was 0.58 s\(^\text{-1}\); for EcRNA\(^{\text{Phe}}\)\text{GAA-G34U} the values were 2.26 \(\mu\)M and 0.50 s\(^\text{-1}\), respectively. These values are similar to those of WT EcRNA\(^{\text{L}}\)\text{C-G34C and -G34U}. Sequence alignment of EcRNA\(^{\text{Ser}}\)\text{CGA} and EcRNA\(^{\text{L}}\)\text{C-G35A 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 T\(\text{D}\)C loop region of EcRNA\(^{\text{Ser}}\)\text{CGA} also differs from that of EcRNA\(^{\text{L}}\)\text{C-G35A} (Fig. 4C). We mutated G35 of EcRNA\(^{\text{Ser}}\)\text{CGA to A in order to mimic the anticodon loop of EcRNA\(^{\text{L}}\)\text{C-G35A} (Fig. 4C). The total levels of isopenetnylation of the plateau by EcMiaA for transcribed tRNA\(^{\text{Ser}}\)\text{CGA and tRNA\(^{\text{Ser}}\)\text{CGA-G35A are the same (Fig. S2). Further methylation assay showed that i\(^\text{A37 modified EcRNA\(^{\text{Ser}}\)\text{CGA-G35A but not}}\)
Anticodon stem minihelices are substrates of \textit{Ec}TrmL

In general, tRNA MTases can be divided into 2 groups based on their sensitivity to structural elements in the tRNA molecule.\textsuperscript{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.\textsuperscript{42} We examined whether the L-shaped structure of tRNA or the anticodon stem loop alone was sufficient for \textit{Ec}TrmL recognition. We constructed several \textit{EctRNA} minisubstrates derived from \textit{EctRNA} (Fig. 5A). The minisubstrates were synthesized by \textit{in vitro} transcription; they all lacked tRNA specific domains including the D-loop, the variable loop and the T\textsubscript{1}C 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 domain with a few nucleotides linking the 2 domains, which originally existed in the D-loop region and the T\textsubscript{1}C region. All the transcripts were modified by recombinant \textit{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 \textsuperscript{3}H labeled DMAPP to ensure that these \textsuperscript{i}A modified tRNA minihelices were used in the same effective concentration in later methyl transfer assays (see Materials and Methods).

The methylation capacities of the \textsuperscript{i}A dimethylallylated minisubstrates revealed that \textsuperscript{i}A-ASL-5 was a poor substrate, but the addition of extra base pairs in \textsuperscript{i}A-ASL-7, \textsuperscript{i}A-ASL-9, \textsuperscript{i}A-ASL-11 and \textsuperscript{i}A-Acc-ASL improved the methylation levels (Fig. 5B,C); these minihelices were methylated to levels comparable to \textsuperscript{i}A modified \textit{EctRNA} or \textit{EctRNA} \textsuperscript{37} transcript. Together, these results showed that an anticodon stem-loop minihelix extension of 2 base pairs is the minimal substrate requirement for \textit{Ec}TrmL methylation.

Only pyrimidines at the wobble position are recognized by \textit{Ec}TrmL

\textit{EctRNA} was the substrate of \textit{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 \textit{Ec}TrmL.

\textit{EctRNA} was the substrate of \textit{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 \textit{Ec}TrmL.

\textbf{Table 1. Kinetic parameters of \textit{Ec}TrmL methylation of various tRNA substrates}

| tRNAs | apparent $K_m$ (\textmu M) | apparent $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ |
|-------|---------------------------|-------------------------------|---------------|
| tRNA\textsuperscript{Leu}\textsubscript{CA} | 3.99 ± 0.33               | 0.44 ± 0.07                | 0.13          |
| tRNA\textsuperscript{Leu}\textsubscript{C34A} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{C34G} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{U34A} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{U34G} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{VLJ} | 3.12 ± 0.30               | 0.71 ± 0.10                 | 0.23          |
| tRNA\textsuperscript{Leu}\textsubscript{VLSD} | 2.74 ± 0.39               | 0.75 ± 0.04                 | 0.27          |
| tRNA\textsuperscript{Leu}\textsubscript{VLAD} | 3.35 ± 0.31               | 0.63 ± 0.09                 | 0.19          |
| tRNA\textsuperscript{Leu}\textsubscript{ASL3} | 4.07 ± 0.26               | 0.85 ± 0.11                 | 0.21          |
| tRNA\textsuperscript{Leu}\textsubscript{ASL31} | 3.39 ± 0.18               | 1.18 ± 0.02                 | 0.35          |
| tRNA\textsuperscript{Leu}\textsubscript{U32C} | 3.54 ± 0.19               | 0.99 ± 0.14                 | 0.28          |
| tRNA\textsuperscript{Leu}\textsubscript{U32A} | 5.37 ± 0.27               | 1.20 ± 0.16                 | 0.22          |
| tRNA\textsuperscript{Leu}\textsubscript{U32G} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{U33A} | 4.46 ± 0.26               | 0.40 ± 0.05                 | 0.09          |
| tRNA\textsuperscript{Leu}\textsubscript{A35U} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{A37C} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Leu}\textsubscript{A38C} | ND                        | ND                           | ND            |
| tRNA\textsuperscript{Ph} \textsubscript{GAA-G34C} | 1.82 ± 0.22               | 0.58 ± 0.05                 | 0.32          |
| tRNA\textsuperscript{Ph} \textsubscript{GAA-G34U} | 2.26 ± 0.15               | 0.50 ± 0.12                 | 0.22          |

\textsuperscript{a}Values from Liu et al.\textsuperscript{22}

The results are the average of three independent experiments with standard deviations indicated.

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\textbf{Figure 4. Alteration of the main tRNA body does not affect recognition by \textit{Ec}TrmL. (A) Secondary structure of \textit{EctRNA} \textsubscript{CA}, highlighting the mutations at position 34 (\textit{EctRNA} \textsubscript{CA-G34A} and \textit{EctRNA} \textsubscript{Ph-G34U}). (B) The capacity of \textit{d} \textit{TrmL- \textit{tRNA} \textsubscript{Ph-G34C} and \textit{d} \textit{TrmL- \textit{tRNA} \textsubscript{Ph-G34U} to be methylated by \textit{Ec}TrmL. (C) Secondary structure of \textit{EctRNA} \textsubscript{CGA}, highlighting the mutation at position 35 (\textit{EctRNA} \textsubscript{CGA-G35A}). (D) The capacity of \textit{A- \textit{Ts-tRNA} \textsubscript{CGA} and \textit{A- \textit{Ts-tRNA} \textsubscript{CGA-G35A} to be methylated by \textit{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\textsubscript{Leu-CAA-C34A} in E. coli.\textsuperscript{16} To determine whether EcTrmL could recognize a purine nucleotide at position 34, we mutated C34 or U34 in the tRNA\textsubscript{Leu} isoacceptors to A34 or G34, resulting in Ec tRNA\textsubscript{Leu-CAA-C34A/G} or Ec tRNA\textsubscript{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 EcMiaA at position 37 in vitro. The methyl transfer assay by recombinant EcTrmL showed no detectable activity when using Ec tRNA\textsubscript{Leu-CAA} or Ec tRNA\textsubscript{Leu-UAA} with purine substitutions at position 34, whether the i\textsuperscript{6}A37 modification was present or not (Fig. 6A,B). This finding shows that only pyrimidine nucleotides at position 34 are substrates of EcTrmL.

**Discussion**

The i\textsuperscript{6}A37 modification has been found to be widespread and conserved.\textsuperscript{43} Defects in this modification were recently found in human pathogenic mutations.\textsuperscript{44} In bacteria, the ms\textsuperscript{2,6}A37 modification is carried out in 2 successive steps by MiaA and MiaB.\textsuperscript{45} It has been shown that the ms\textsuperscript{2,6}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.\textsuperscript{40} In E. coli, both tRNA\textsubscript{Leu-CAA} and tRNA\textsubscript{Leu-UAA} isoacceptors contain the ms\textsuperscript{2,6}A37 modification. Interestingly, in vitro transcribed tRNA\textsubscript{Leu-CAA} and tRNA\textsubscript{Leu-UAA} without modifications are not substrates of TrmL methylase, suggesting a sequential process starting with the ms\textsuperscript{2,6}A37 modification.\textsuperscript{20} We show here that synthetic transcripts of tRNA\textsubscript{Leu-CAA} and tRNA\textsubscript{Leu-UAA} with the first i\textsuperscript{6}A37 modification, catalyzed by recombinant MiaA, are substrates of TrmL. Similarly, tRNA\textsubscript{Leu} purified from an E. coli MiaA-knockout strain was an effective substrate of TrmL after in vitro introduction of i\textsuperscript{6}A37 modification by recombinant MiaA. These data demonstrate that only the i\textsuperscript{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\textsuperscript{2,6}A37 base.\textsuperscript{16} Altogether, the results confirm that the modifications of

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**Figure 5.** Truncated tRNA minihelices could be recognized by EcTrmL. (A) Secondary structure of the minihelices of the ASL of Ec tRNA\textsubscript{Leu-CAA} (ASL5/7/9/11 and Acc-ASL). (B) and (C) show the capacity of the minihelices to be methylated by EcTrmL. 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\textsuperscript{Leu}_{\text{CAA}} and tRNA\textsuperscript{Leu}_{\text{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 pyrimidines at the wobble position could be methylated by TrmL, which is consistent with previous results showing that TrmL could not methylate Ec \textit{tRNA}_{\text{Leu}}\textsuperscript{CAA-C34A}.\textsuperscript{16} Ec \textit{tRNA}_{\text{Phe}}\textsuperscript{GAA-G34C} and Ec \textit{tRNA}_{\text{Phe}}\textsuperscript{GAA-G34U} mutants were methylated by EcTrmL as efficiently as Ec \textit{tRNA}_{\text{Leu}}\textsuperscript{CAA}.

Our results also showed that TrmL could accommodate many changes in the tRNA\textsuperscript{Leu}_{\text{CAA}} structure. For instance, changes in the variable loop of \textit{EtRNA}_{\text{Leu}}\textsuperscript{CAA} do not affect TrmL activity. Mutations of U32 and U33 preserved \textit{EtTrmL} methylation activity. The nucleotide at position 32 in \textit{E. coli} tRNAs is generally a semi-conserved cytidine or uridine residue\textsuperscript{46} and U33 is invariant in all tRNAs.\textsuperscript{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 \textit{EtTrmL} recognition, which is consistent with the results that TrmL could not methylate tRNA\textsuperscript{Leu}_{\text{CAA-A35U}} in \textit{E. coli}.\textsuperscript{16} The tRNA mutant A36C could not be overexpressed in \textit{E. coli} JW3581-1 in vivo, neither its transcript could be recognized by EcMiaA in vitro. The tRNA mutants A37C and A38C resulted in the loss of the \textit{i}\textsuperscript{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 \textit{i}\textsuperscript{6}A37 modification. Therefore, the reason for these mutants could not be recognized by TrmL may be the loss of \textit{i}\textsuperscript{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 \textit{EtRNA}_{\text{Ser}}\textsuperscript{CGA-G35A} could not be overexpressed in the TrmL gene deletion strain. So we transcribed it by T7 RNA polymerase and modified by EcMiaA at position 37 in vitro. Our results show that \textit{EtRNA}_{\text{Ser}}\textsuperscript{CGA-G35A} turns EtRNA\textsuperscript{Ser}_{CGA} into a substrate for TrmL. Although this mutant could be recognized by TrmL, the efficiency of \textit{EtTrmL} to methylate \textit{EtRNA}_{\text{Ser}}\textsuperscript{CGA-G35A} is rather low compared with \textit{i}\textsuperscript{6}A37 modified \textit{EtRNA}_{\text{Leu}}\textsuperscript{CAA} transcripts. Because main body of tRNA, including anticodon stem, amino acid acceptor stem, the variable loop region, the D loop region and the T\textit{C} loop region of \textit{EtRNA}_{\text{Ser}}\textsuperscript{CGA} differ from that of \textit{EtRNA}_{\text{Leu}}\textsuperscript{CAA}, the reason for \textit{EtRNA}_{\text{Ser}}\textsuperscript{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 EcTrmL could modify tRNA minihelices exhibiting the anticodon stem-loop structure (ASL) and the i^6A37 modification catalyzed by EcMiaA.

In summary, these results suggest that EcTrmL 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 EcTrmH, 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 EcTrmJ catalyzes 2'-O-methylation at position 32 of tRNA.38 EcTrmJ 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 tRNA, mRNA, tRNA, microRNA and other small RNAs. Recently, RNA demethylases were identified that
were found to be involved in fundamental regulatory roles in many important life processes, including germline development, cellular signaling, and circadian rhythm control. 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, 7-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-nitrotriacetic (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 enzymes of the methylation reactions of E. coli tRNAs were made by direct primer annealing. The A37-dimethylallyl diphosphate (DMAPP) in the \(^{3}H\) 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 concentrations of transcribed minihelices, reactions were performed under identical conditions at 37°C for 20 min before quenching by phenol extraction. To measure the effective concentrations of transcribed minihelices, reactions were performed using a range of 0.5–20 μM tRNA, 100 μM SAM and 0.2 μM TrmL.

Preparation of tRNAs and mutagenesis

The genes encoding EctRNAs including EctRNA^{Lens}, EctRNA^{Leu}_{UAU}, EctRNA^{Phe}_{GAU} were amplified from the genome of E. coli MG1655. Site-directed mutagenesis of EctRNAs was performed using the KOD-plus mutagenesis kit as described previously. 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. Unmodified tRNAs were made by in vitro transcription by T7 RNA polymerase according to previous protocols. The tRNA minihelices were synthesized by in vitro transcription of DNA templates that were made by direct primer annealing. The A37-dimethylallyl-modified (i\(^6\)A37) tRNAs were made using recombinant E. coli MiaA (EcMiaA) 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.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out as previously described. Then, 80 nM tRNA with a range of 0.75–10 μM EctRNAs 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.

www.tandfonline.com RNA Biology 909

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Supplemental Material

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