Supplementary material

Identification of a novel tRNA wobble uridine modifying activity in the biosynthesis of 5-methoxyuridine

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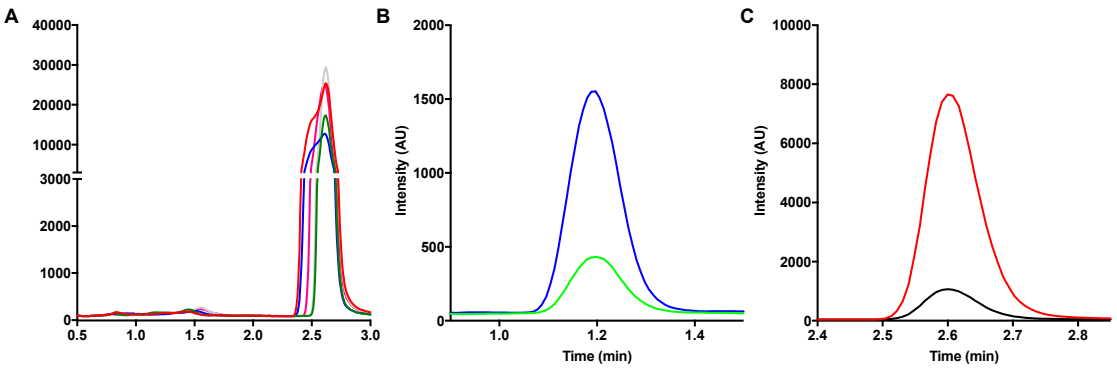
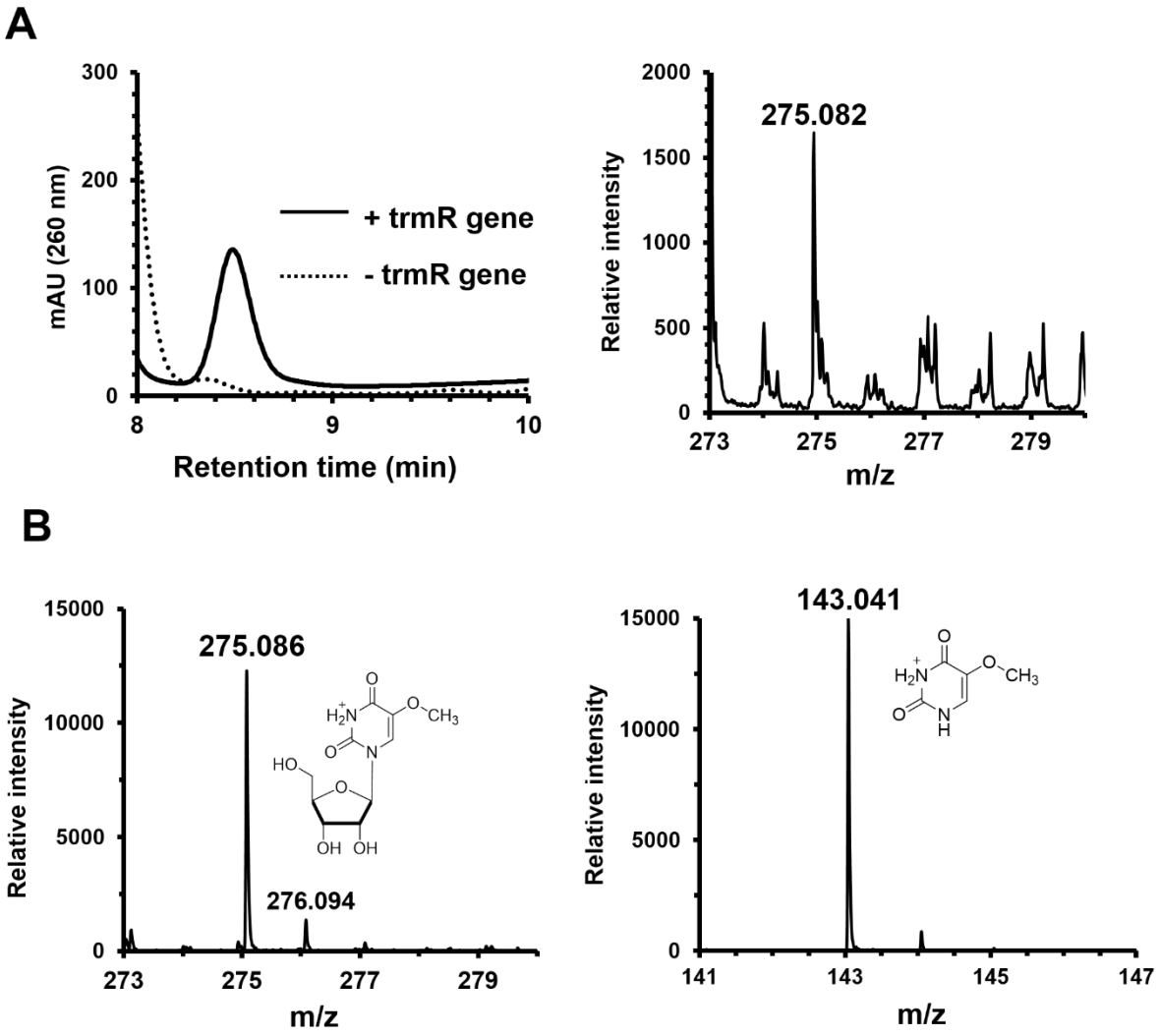
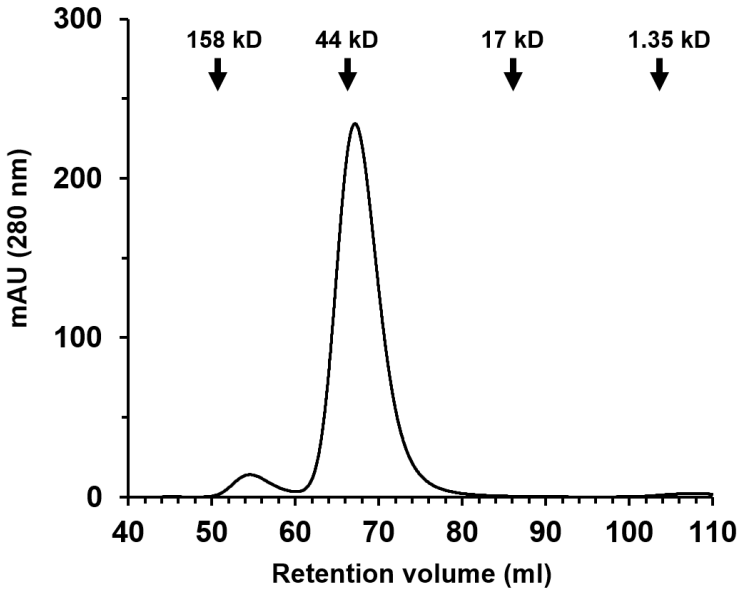


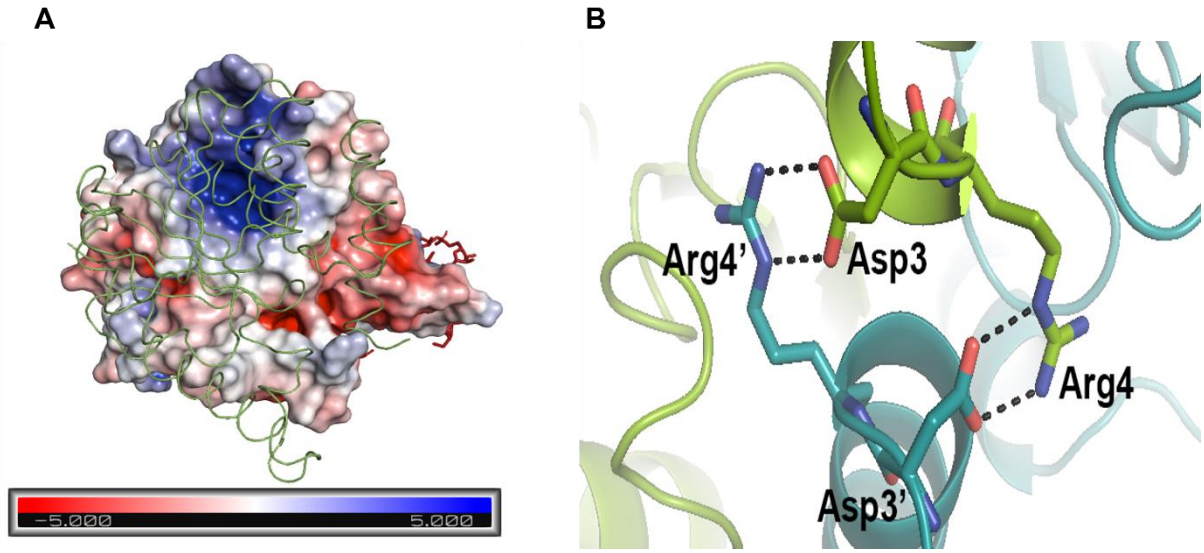
Supplementary Figure 1. *In vivo* production of mo5U by *B. subtilis* deletion strains. **A)** The presence of mo^{5}U in total RNA extracted from *B. subtilis* and the single knockout mutants was examined by LC-MS. The Δ*yodH,* Δ*ydaC,* Δ*yibH,* Δ*yrhH,* Δ*ycgJ,* Δ*yacO, and* Δ*yrrT* strains of *B. subtilis* all produce mo^{5}U (peak at 2.7 min). **B**) LC-MS MRM parent peak to product peak transitions used to monitor ho^{5}U. The blue trace is the *m/z* transition of 261.1 to 129.2. The green trace is the *m/z* transition of 261.1 to 133.1. **C**) LC-MS MRM transitions used to monitor mo^{5}U. The red trace is the *m/z* transition of 275.1 to 143.1. The black trace is the *m/z* transition of 275.1 to 97.8.



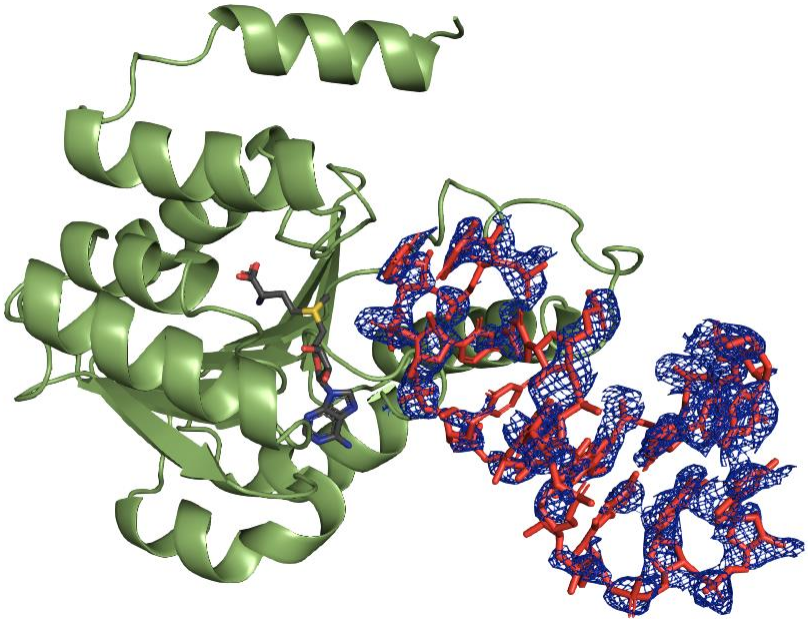
Supplementary Figure 2. Substrate specificity of TrmR against *E. coli* tRNA. (A) (left) *In vivo* ho^{5}U methylation was confirmed by analyzing HPLC trace of mo^{5}U from total RNA, extracted from *cmoB*-deficient *E. coli* cells complemented with *trmR*-expressing plasmid (+ trmR). RNAs from mutant cells complemented with an empty vector (- trmR) were analyzed as negative control. (right) The *m/z* peak corresponding to mo^{5}U ($[C_{10}H_{15}O_7N_2]^+$, m/z = 275.08) was observed in + trmR sample only by LC-MS. (B) *In vitro* methylation of ho5U-containing tRNAs from *E. coli* was confirmed by LC-MS/MS analysis, where the parent peak (left), and its major fragment peak (right), 5-methoxyuracil ($[C_5H_7N_2O_3]^+$, m/z = 143.04), are shown.



Supplementary Figure 3. Representative gel filtration profile of purified recombinant *B. subtilis* TrmR. Arrows indicate the elution volumes of standard proteins; Vitamin B12 (1.35 kD), Myoglobin from horse (17 kD), Ovalbumin from chicken (44 kD) and bovine γ-globulin (158 kD). Elution volume of *B.subtilis* TrmR is estimated to be 64 ± 0.8 ml (an average ± standard deviation from three measurements).



Supplementary Figure 4. Homodimeric interface within TrmR-ASL^{Ala} structure (A) One subunit is presented with solvent accessible surface, whereas the other subunit is shown as backbone trace in green for clear visualization of the dimeric interface. Distribution of electrostatic potential is mapped on the protein surface. Color bar of blue, red and white are indicated positive, negative and neutral electrostatic potential. (B) Salt bridge at the dimeric interface between a pair of Asp-3 and Arg-4.



Supplementary Figure 5. Omit electron density map of tRNA. Fourier difference map (*Fo* - *Fc*) was calculated without modeling tRNA during refinement and displayed as blue mesh contoured at 2.0 sigma.

A.

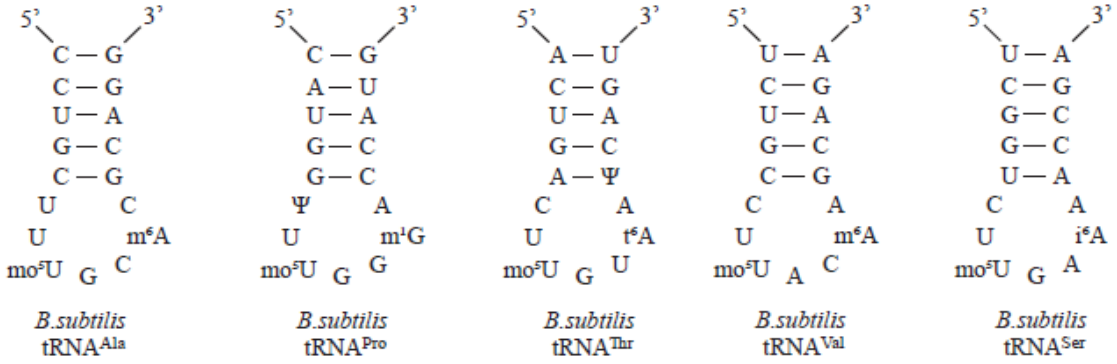
Four Box tRNA Sets

Isotype	tRNA Count by Anticodon				Total
Ala	AGC	GGC 1	CGC	TGC 5	6
Pro	AGG	GGG	CGG	TGG 3	3
Thr	AGT	GGT 1	CGT	TGT 4	5
Val	AAC	GAC 1	CAC	TAC 4	5

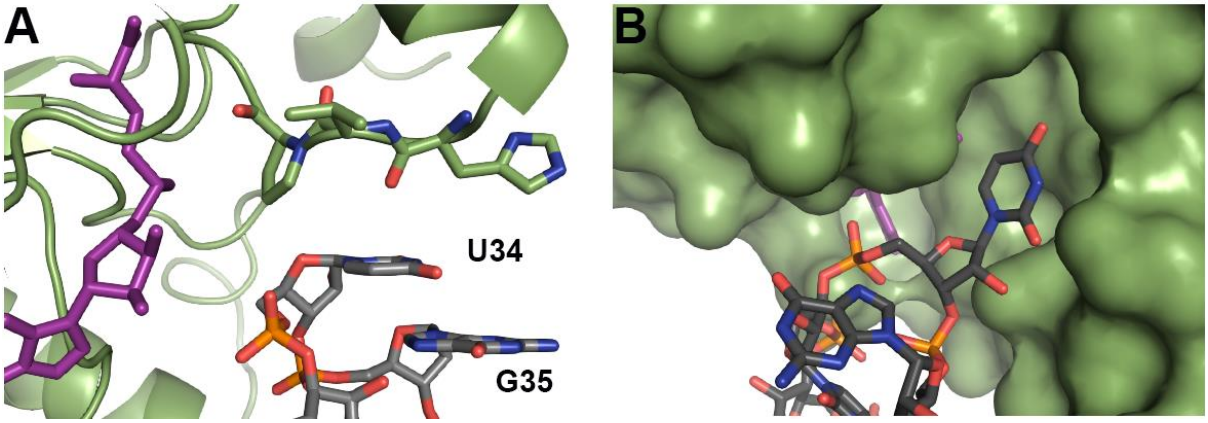
Six Box tRNA Sets

Isotype	tRNA Count by Anticodon						Total
Ser	AGA	GGA 1	CGA	TGA 2	ACT	GCT 2	5

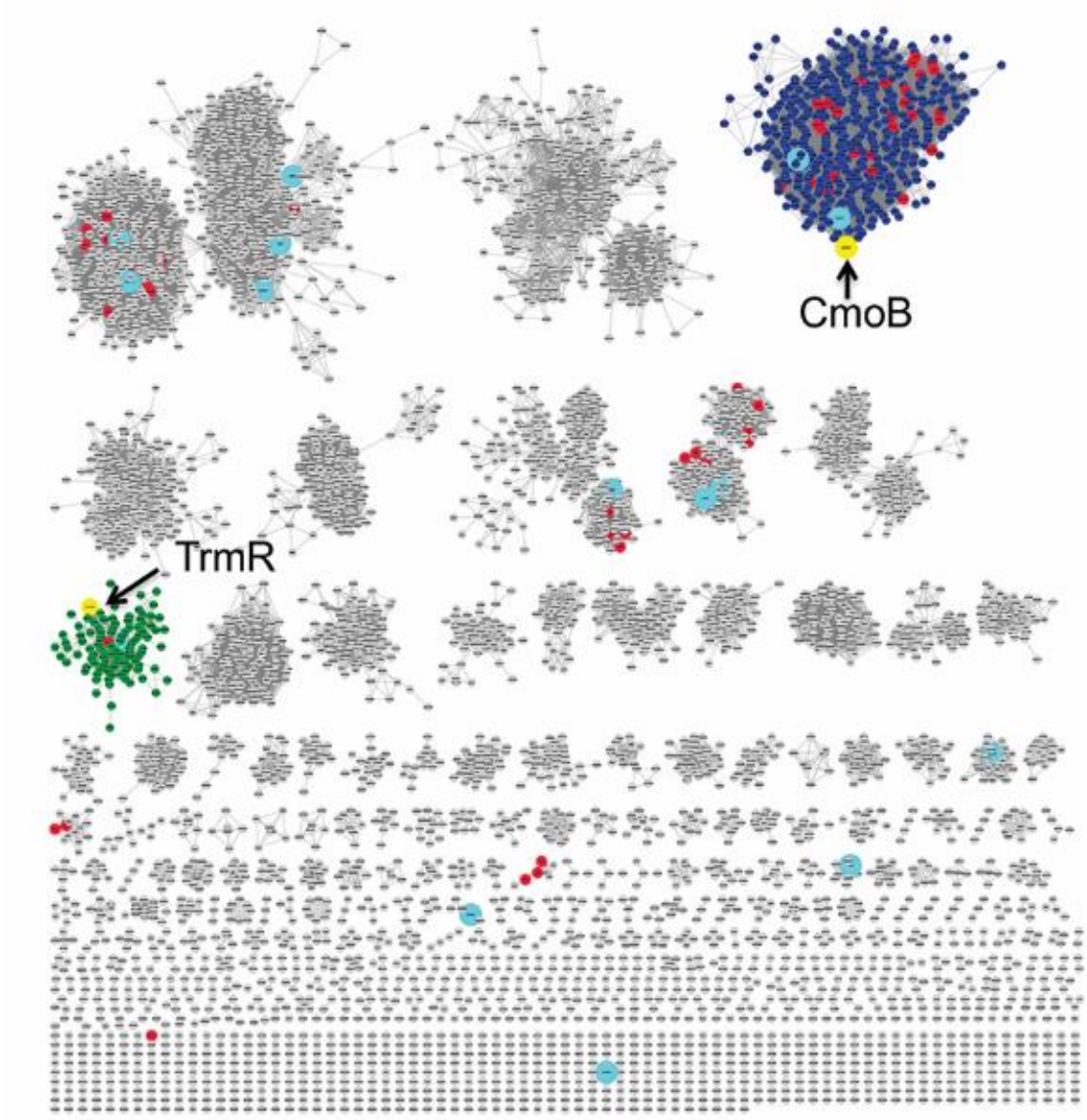
B.



Supplementary Figure 6. (A) Number of copies of tRNA genes for Ala-, Pro-, Thr-, Val- and Ser-specific isoacceptors in *B. subtilis* genome. Anticodons to be modified to mo5U are highlighted in red. (B) Secondary structures of anticodon stem loops with mo^{5}U34 in *B. subtilis.*



Supplementary Figure 7. Conformation of the wobble uridine in the TrmR active site. A) In TrmR-ASL^{Ala} structure, the wobble uridine is sandwiched between G35 and a short peptide loop (His-34, Val-35, and Pro-36), resulting in an inactive conformation. B) In 'base-flipped' model, uracil base of wobble uridine is in attack-ready conformation. Nearby SAM is presented in purple.



Supplementary Figure 8. Presentation of sequence similarity network of PF08003 and PF01596. Members of Methyltransf_9 (PF08003, which includes CmoB) and Methyltransf_3 (PF01596, which includes TrmR) were combined and their sequence similarity was analyzed. After filtering peptides smaller than 150 amino acids, a total of 24,846 sequences were aligned using EFI-Enzyme Similarity Tool (https://efi.igb.illinois.edu/efi-est/) at the E-value of -60, and RepNode of 50%, which resulted in 5,164 nodes and 93,823 edges. Nodes in 'TrmR cluster' are highlighted in green, whereas those in 'CmoB cluster' are in navy. Red nodes indicate Swiss-Prot annotated sequences, and teal nodes contain sequences with a structure. Nodes containing TrmR and CmoB are labeled in yellow. MdmC and caffeoyl-CoA-O-MTase described in the main text are located in the upper left cluster.