

Pseudouridine at position 55 in tRNA controls the contents of other modified nucleotides for low-temperature adaptation in the extreme-thermophilic eubacterium *Thermus thermophilus*

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ABSTRACT

Pseudouridine at position 55 (Ψ 55) in eubacterial tRNA is produced by TruB. To clarify the role of the Ψ 55 modification, we constructed a *truB* gene disruptant (Δ *truB*) strain of *Thermus thermophilus* which is an extreme-thermophilic eubacterium. Unexpectedly, the Δ *truB* strain exhibited severe growth retardation at 50°C. We assumed that these phenomena might be caused by lack of RNA chaperone activity of TruB, which was previously hypothetically proposed by others. To confirm this idea, we replaced the *truB* gene in the genome with mutant genes, which express TruB proteins with very weak or no enzymatic activity. However the growth retardation at 50°C was not rescued by these mutant proteins. Nucleoside analysis revealed that Gm18, m⁵s²U54 and m¹A58 in tRNA from the Δ *truB* strain were abnormally increased. An *in vitro* assay using purified tRNA modification enzymes demonstrated that the Ψ 55 modification has a negative effect on Gm18 formation by TrmH. These experimental results show that the Ψ 55 modification is required for low-temperature adaptation to control other modified. ³⁵S-Met incorporation analysis showed that the protein synthesis activity

of the Δ *truB* strain was inferior to that of the wild-type strain and that the cold-shock proteins were absent in the Δ *truB* cells at 50°C.

INTRODUCTION

Pseudouridine (Ψ) is the most abundant modified nucleoside in RNA and is frequently found in tRNA, rRNA, snRNA, snoRNA and tmRNA (1–6). Ψ is formed post-transcriptionally via C5-ribosyl isomerization of uridine by a class of enzymes known as Ψ synthases. Most Ψ synthases recognize one or a few target uridines and catalyze the isomerization.

Transfer RNA (Ψ 55) synthase [EC.5.4.99.12 (7); TrpU(Y55) (1)] catalyzes Ψ 55 formation in the T-loop of tRNA. The Ψ 55 modification is commonly found in eubacterial, archaeal, eukaryotic and organelle tRNA (1–3). The Ψ 55 forms a tertiary base pair with the conserved G18 in D-loop and stabilizes the L-shaped tRNA structure (8,9). Eubacterial tRNA (Ψ 55) synthase is one of the best studied tRNA modification enzymes. The Ψ formation activity towards a tRNA precursor was first detected in *Escherichia coli* cell extract (10) and subsequently tRNA (Ψ 55) synthase activity was separated from tRNA (Ψ 38, 39, 40) synthase activity (11). The tRNA (Ψ 38, 39, 40) synthase is now called tRNA Ψ synthase I (TruA) (7,12–14). To date, three tRNA Ψ

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synthases (TruC, RluA and TruD) have been additionally found in *E. coli* (15,16). *E. coli* tRNA (Ψ 55) synthase was purified to near homogeneity as judged by SDS–polyacrylamide gel analysis, and found to be encoded by the *truB* gene (17). Therefore, hereafter we describe eubacterial tRNA (Ψ 55) synthase as TruB. It should be mentioned that the Ψ 55 in archaeal tRNA is formed by Cbf5 (18–20) and Pus10 (18,21), and that Ψ 55 in eukaryotic cytoplasmic and mitochondrial tRNA is formed by Pus4 (22). Although the *truB* genes are found in almost all eubacterial genomes (23,24), the gene has been experimentally identified in *E. coli* (17), *Bacillus subtilis* (17), *Shigella flexneri* (25), *Pseudomonas aeruginosa* (25,26) and *Thermotoga maritima* (27,28). TruB recognizes the T-arm structure and can modify the target uridine in a 17-mer T-arm fragment (29). X-ray crystal structures of TruB and TruB-RNA complex have been studied and have revealed that TruB undergoes significant conformational changes upon binding to RNA substrate (27,30–32). Based on amino acid sequence alignment and crystal structures, the mechanism of uridine isomerization has also been studied (23,24,28,30,31,33–36) and the catalytic residue of TruB been identified as a conserved aspartic acid (34). Thus, *in vitro* protein and RNA chemistry studies have made significantly progress in the past 15 years since the identification of the *truB* gene.

In vivo functional study of the Ψ 55 modification in tRNA is not straightforward because a *truB* deletion mutant generally grows normally. For example, deletion of *E. coli truB* does not affect exponential growth (37) and the lack of the Ψ 55 modification does not cause frameshift error on translational ribosomes in *E. coli* cells (38). However, a large effort has been made toward elucidating the importance of TruB protein and the Ψ 55 modification. Thus, the translational activity of nine-repeated CGA codons in the *E. coli truB* deletion mutant is inferior to that in the wild-type strain (25). The *E. coli truB* deletion mutant exhibits a defect in survival of rapid transfer from 37°C to 50°C (39). Deletion of *truB* in *S. flexneri* reduces the expression of some virulence-associated genes (25). Disruption of *truB* (classical name, *orp* gene) by Tn5 in *P. aeruginosa* results in impaired growth on BHI plates at 43°C and a reduced amounts of virulence factor phospholipase C compared to the wild-type strain (25,26). Deletion of *truB* alone in *E. coli* does not affect the suppressor tRNA activity of *tyrT* (*supF*), however increased suppression ability in *truB–trmH* and *truB–trmA* double deletion mutants is observed (25). Furthermore, these double deletion mutants show reduced growth (25). The genes, *trmH* and *trmA* encode tRNA (Gm18) methyltransferase (24,40–46) and *S*-adenosyl-L-methionine (AdoMet)-dependent tRNA (m^5U54) methyltransferase (47–51), respectively. These facts suggest that Ψ 55 modification probably works with other modifications (Gm18 and m^5U54) at the elbow region in the L-shaped tRNA structure and that Ψ 55 modification and/or TruB protein itself is required for stress resistance. In these functional studies, a very interesting hypothesis has been proposed namely that TruB protein may act as an RNA chaperone to assist in correct folding of tRNA (37).

In this article, we found that a *truB* disruptant strain of *Thermus thermophilus*, an extreme-thermophilic eubacterium, showed growth retardation at low temperatures (below 60°C). We demonstrate that the lack of the Ψ 55 modification causes abnormal enhancement of the amount of other modified nucleotides. We describe in this article that the Ψ 55 modification is required for low temperature adaptation for the organism. Furthermore, the RNA chaperone hypothesis of the TruB protein is discussed.

MATERIALS AND METHODS

Materials

[α - ^{32}P]-Uridine 5'-triphosphate (222 TBq/mmol; 0.37 MBq/ μ l) was purchased from MP Biomedicals. [Methyl- ^{14}C]-AdoMet (1.95 GBq/mmol) and [methyl- 3H]-AdoMet (2.47 TBq/mmol) were purchased from Perkin Elmer. L-[^{35}S]-Methionine (37 TBq/mmol) was purchased from Muromachi Yakuhin. DE52 is a product of Whatman. CM-Toyopearl 650 M was bought from Tosoh. DNA oligomers were obtained from Invitrogen and T7 RNA polymerase was from Toyobo. Other chemical reagents were of analytical grade.

Strain and media

Culture source of *T. thermophilus* HB8 was a kind gift from Dr Tairo Oshima (Tokyo University of Pharmacy and Life Science). The cells were grown in rich medium [0.8% polypeptone, 0.4% yeast extract and 0.2% NaCl, pH 7.5 (adjusted with NaOH)]. The medium was supplemented with 0.35 mM CaCl₂ and 0.17 mM MgCl₂ after autoclaving. To make plates, gellan gum (Wako Pure Chemicals) was added to the medium (final concentration, 1.5%).

Selection of a target gene for gene disruption and preparation of the recombinant TruB protein

The TTHA0217 gene in *T. thermophilus* HB8 genome has been annotated as *truB* by the *T. thermophilus* DNA sequencing project (52). A DNA fragment of the TTHA0217 gene was amplified by PCR using following primers: Tth TruBF, 5'-CCC CCA TAT GGC CCT CTA CGC GGT GGA CAA G-3'; Tth TruBR, 5'-CCC CGG ATC CTT ACG CCT CCT TCG CAA ACA CGC C-3'. Underlining shows restriction enzyme sites (Nde I and Bam HI). The amplified DNA was digested with Nde I and Bam HI, and ligated into the multi-cloning linker of pET30a *E. coli* expression vector. The expression of TTHA0217 protein in *E. coli* BL21 (DE3) Rosetta 2 strain (Novagen) was performed according to the manufacturer's manual. The protein was purified by DE52 column chromatography, heat-treatment and CM-Toyopearl 650 M column chromatography. Briefly, the cells (5g) were suspended in 25 ml of buffer A [50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 6 mM 2-mercaptoethanol and 50 mM KCl] and disrupted with an ultrasonic disruptor model UD-200 (Tomy, Japan). The cell debris was removed by centrifugation at 8000g for 20 min. The supernatant fraction was applied onto a

DE52 column (column volume, 10 ml). The flow-through fractions were collected and then heated at 70°C for 30 min. The denatured proteins were removed by centrifugation at 8000g for 20 min. The supernatant fraction was dialyzed against buffer B [50 mM Hepes-KOH (pH 6.8) and 6 mM 2-mercaptoethanol]. The dialyzed sample was loaded onto a CM-Toyopearl 650M column (column volume, 10 ml) and TruB protein eluted with buffer B containing 100–150 mM KCl. The eluted sample was dialyzed against buffer A and concentrated with Centriprep YM-10 centrifugal filter devices (Millipore). Glycerol was added to the purified protein to a final concentration of 50% v/v and stored at –30°C. Quantification of protein was performed with a Bio-Rad protein assay kit using bovine serum albumin as standard.

Measurement of Ψ formation activity

We selected *T. thermophilus* tRNA^{Thr} (GGT) as the substrate of TruB because this tRNA is expected to possess only one Ψ modification at position 55 (2). Template DNA for transcription was prepared with the following primers: Tth tRNA^{Thr} F, 5'-TAA TAC GAC TCA CTA TAG CTC GCG TAG CTC AGC AGG TAG AGC ACA CCC TTG TAA GGG TG-3'; Tth tRNA^{Thr} R, 5'-TGG AGC TCG CGG CCG GGC TCG AAC CGG CCC TCA CGC TTA CCA AGG GTG TG-3'. Run-off transcription by T7 RNA polymerase was performed according to our previous reports (42,53). In the case of internal labeling, 2 μ l of α -³²P-UTP was added into the reaction mixture. The transcript was purified by 10% PAGE (7 M urea). About 0.4 A₂₆₀ units of tRNA transcript (containing ³²P around 18000 dpm) and 2 μ g of the purified TruB protein in 40 μ l buffer A were incubated at 55°C for 1 h. The RNA was extracted with phenol–chloroform and then recovered by ethanol precipitation. The RNA pellet was dissolved in 3 μ l of 50 mM sodium acetate (pH 5.0) and digested with 2.5 U of nuclease P1 (Wako Pure Chemicals). The sample was separated by two dimensional thin layer chromatography (2D-TLC) as described previously (54). The ³²P-labeled nucleotides were monitored with a Fuji Photo Film BAS2000 imaging analyzer.

Construction of Δ *truB* (Δ TTHA0217) strain

The TTHA0217 gene was disrupted by replacement with the highly thermostable kanamycin nucleotidyltransferase (*HTK*) gene (55,56). The plasmid vector containing the TTHA0217 region disrupted by the *HTK* gene was purchased from RIKEN Biological Resource Center (Tsukuba, Japan) (52). *Thermus thermophilus* cells in late-log phase were transformed by the vector according to the report of Hashimoto *et al.* (57) and mutant colonies were selected on a plate containing 500 μ g/ml kanamycin at 70°C. The genomic DNA from each colony was isolated, analyzed by PCR and then the sequence of the replaced region was determined on ABI PRISM 310 DNA sequencers.

Purification of native tRNA^{Metf1} by solid-phase DNA probe

The 3'-Biotinylated DNA oligomer (5'-GGG TTA TGA GCC CGA CGA GCT ACC –biotin-3') was used as a hybridization probe. The probe sequence is complementary from C41 to G19 of *T. thermophilus* tRNA^{Metf1}. Before solid phase DNA probe column chromatography, tRNA^{Metf1} fraction was prepared by BD-cellulose column chromatography as reported previously (58). Purification of tRNA^{Metf1} by solid-phase DNA probe was performed as described in our recent reports according to the suggestions of Dr Yokogawa (Gifu University) (59–61). The eluted tRNA^{Metf1} was further purified by 10% PAGE (7 M urea).

Nucleosides analysis by HPLC

Nucleoside analysis by HPLC was performed according to our recent report (60).

Construction of *T. thermophilus* *truB* mutant strain

Site-directed mutagenesis was performed using the Quick change mutagenesis kit (Stratagene). Schemes of construction of plasmid vectors for gene replacement of the *truB* mutant genes are shown in Supplementary Figure S3. The homologous recombination was performed as described above. The genomic DNA was isolated, analyzed by PCR and then the sequence of the replaced region was determined on ABI PRISM 310 DNA sequencers.

Western blotting analysis

Customized rabbit anti-*T. thermophilus* TruB serum was prepared by Oriental Kobo Co., Ltd, Japan. The polyclonal antibody fraction was partially purified using the Econo-pac serum IgG purification kit (Bio-Rad). Cultured cells were directly resuspended in SDS–PAGE sample buffer and then disrupted. The samples were boiled and immediately loaded onto a 15% SDS–polyacrylamide gel. Electro-blotting to a nitrocellulose membrane (Nitro Bind membrane code EP2HY315F5, pore size 0.45 μ m, Osmonics) was performed using a semi-dry blotting system (NA-1515B, Nippon Eido) according to the manufacturer's instructions. TruB protein was detected using Alexa Fluor 488 anti-rabbit IgG (Invitrogen) as a secondary antibody and visualized using a Typhoon model 9410 (GE healthcare).

Preparation of tRNA modification enzymes

Thermus thermophilus tRNA (Gm18) methyltransferase (TrmH) and *Aquifex aeolicus* tRNA (m⁷G46) methyltransferase (TrmB) were purified according to our previous reports (41,62). *Thermus thermophilus* tRNA (m¹A58) methyltransferase (TrmI) (63) was a gift from Masayuki Minoji (Ehime University). *Escherichia coli* tRNA (m⁵U54) methyltransferase (TrmA) (47–49) was expressed in *E. coli* and purified as follows. TrmA coding region was amplified by PCR from *E. coli* BL21 (DE3) genomic DNA using the following primers: EcTrmAN, 5'-CCC CCA TAT GAC CCC CGA ACA CCT TCC ACC-3'; EcTrmAC, 5'-CCC CGG ATC CTT ACT TCG

CGG TCA GTA ATA CGC CGC ACT CCA TGT GGT GC-3'. Underlining shows restriction enzyme sites (Nde I and Bam HI). Double underlining shows a mutation site for disruption of the Nde I site, which exists in the original *E. coli* genomic DNA. This mutation does not change the amino acid sequence of TrmA. The amplified DNA was digested with Nde I and Bam HI, and ligated into the multi-cloning linker of pET30a *E. coli* expression vector. The expression of TrmA protein in *E. coli* BL21 (DE3) Rosetta 2 strain was performed according to the manufacturer's manual. Cells (4.5g) were suspended in 20 ml of buffer C [10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM DTT and 10% glycerol] and then disrupted with an ultrasonic disruptor model UD-200 (Tomy, Japan). The cell debris was removed by centrifugation at 8000g for 20 min and then ammonium sulfate was added to the supernatant fraction (final concentration, 1.7 M). The sample was further centrifuged at 24000g for 20 min. The supernatant fraction was passed through a Steradisc 25 filter (0.2 μm, Kurabo) and then loaded onto a HiPrep Phenyl FF low sub 16/10 column (GE Healthcare). A linear gradient was developed from 1.7 M to 0 M ammonium sulfate in buffer C. TrmA fractions were assessed by 15% SDS-PAGE, combined, dialyzed against buffer A and concentrated with Centriprep YM-10 centrifugal filter devices (Millipore). Glycerol was added to the purified protein to a final concentration of 50% v/v and stored at -30°C.

In vitro tRNA modification enzyme assay

To estimate the content of unmodified nucleotides in tRNA, tRNA mixtures from the wild-type and *ΔtruB* strains were methylated with purified tRNA modification enzymes. The tRNA mixtures were prepared from the cells cultured at 50°C. About 0.2 A₂₆₀ units of tRNA mixture, enzyme (45.0 pmol TrmH, 28.6 pmol TrmB or 17.5 pmol TrmI) and 19 μM ¹⁴C-AdoMet were incubated in 40 μl buffer A at 55°C over night. In the case of TrmA (57 pmol), the incubation temperature was 37°C. The ¹⁴C-methyl group incorporation was monitored by a liquid scintillation counter using a filter assay. For the time-course assay, *T. thermophilus* class I tRNA from the *ΔtruB* strain with the Ψ55 modification was prepared as follows. About 1.63 A₂₆₀ units of class I tRNA from the *ΔtruB* strain was incubated with 1.0 nmol wild-type TruB in 100 μl buffer A at 55°C for 3 h. The sample was extracted with phenol and the RNA was recovered by ethanol precipitation. We checked the purity of the recovered transcript by 10% PAGE (7 M urea). The time-course assay was performed as follows: 0.1 A₂₆₀ units of class I tRNA fraction with or without Ψ55 modification, 26 μM ³H-AdoMet and 45.0 pmol TrmH were incubated in 30 μl buffer A at 55°C.

Melting profile analyses of class I tRNA

Class I tRNA fractions from the wild type and *ΔtruB* strains cultured at 50°C and 70°C were prepared as described above. Before the melting point measurement the tRNA fraction was annealed in buffer D [50 mM Tris-HCl (pH7.5), 5 mM MgCl₂, 100 mM NaCl] from 80°C to 40°C for 60 min and then the melting curve

was recorded on a spectrophotometer, UV-1650PC (Shimadzu). The temperature was increased from 40 to 95°C over 55 min. The melting profiles were obtained by averaging two scans. The melting temperatures were calculated from first derivative plots.

Translation activity analyses by ³⁵S-Met incorporation

The wild-type and *ΔtruB* strains were cultured at 50 or 70°C. When the cell density (A_{600 nm}) had reached 0.6, the culture medium (60 ml) was supplemented with 12.0 MBq of ³⁵S-Met. The sample (15 ml each) was taken at 0, 15, 30 and 60 min. The cells were collected by centrifugation at 3500g for 5 min. The cells were washed with 5 ml of buffer A and collected by centrifugation at 3500g for 5 min. The cells were resuspended in 120 μl of buffer A after which 120 μl of SDS loading buffer [100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 2.5% SDS, 0.2% bromophenol blue, 20% glycerol] was added. The sample was boiled for 10 min and centrifuged at 21 500g for 10 min. The supernatants were analyzed by 15% SDS-PAGE. The gels were stained with Coomassie brilliant blue and ³⁵S-Met incorporation was monitored with a Fuji Photo Film BAS2000 imaging analyzer.

RESULTS

The Ψ55 modification is absent in the purified tRNA^{Metf1} from the *ΔTTHA0217* strain

As shown in Figure 1A, the RNA sequence of *T. thermophilus* tRNA^{Metf1} has been reported and this tRNA has only one Ψ modification at position 55 (58). In order to check whether the TTHA0217 gene is *truB*, we purified this tRNA by solid phase DNA probe column chromatography (59–61). *Thermus thermophilus* has two initiator tRNA^{Met} species, tRNA^{Metf1} and tRNA^{Metf2} (58). Because the only difference between these initiator tRNA^{Met} species is one base pair in their T-stem, separation of these tRNA species by DNA probe proved impossible. Therefore, we initially separated two initiator tRNA^{Met} species by BD-cellulose column chromatography as described (58) and then the tRNA^{Metf1} rich fraction was used for the solid phase DNA probe column chromatography. We successfully purified tRNA^{Metf1} from the wild-type and *ΔTTHA0217* strains (Figure 1B). The modified nucleosides of these tRNA species were analyzed by HPLC C18 reverse phase column chromatography (Figure 1C). The Ψ nucleoside was eluted at 6.7 ml on our HPLC system. As shown in Figure 1C (upper panel), the Ψ nucleoside was clearly observed in the wild-type strain sample. In contrast, the peak of Ψ nucleoside was missing in the tRNA^{Metf1} from the *ΔTTHA0217* strain (Figure 1C, lower panel), demonstrating that the TTHA0217 gene is *T. thermophilus truB*. Hereafter, we describe *ΔTTHA0217* as *ΔtruB*.

Growth retardation of the *ΔtruB* strain at low temperatures

We examined the growth phenotype of the *ΔtruB* strain at various temperatures. Figure 2 shows the results of liquid

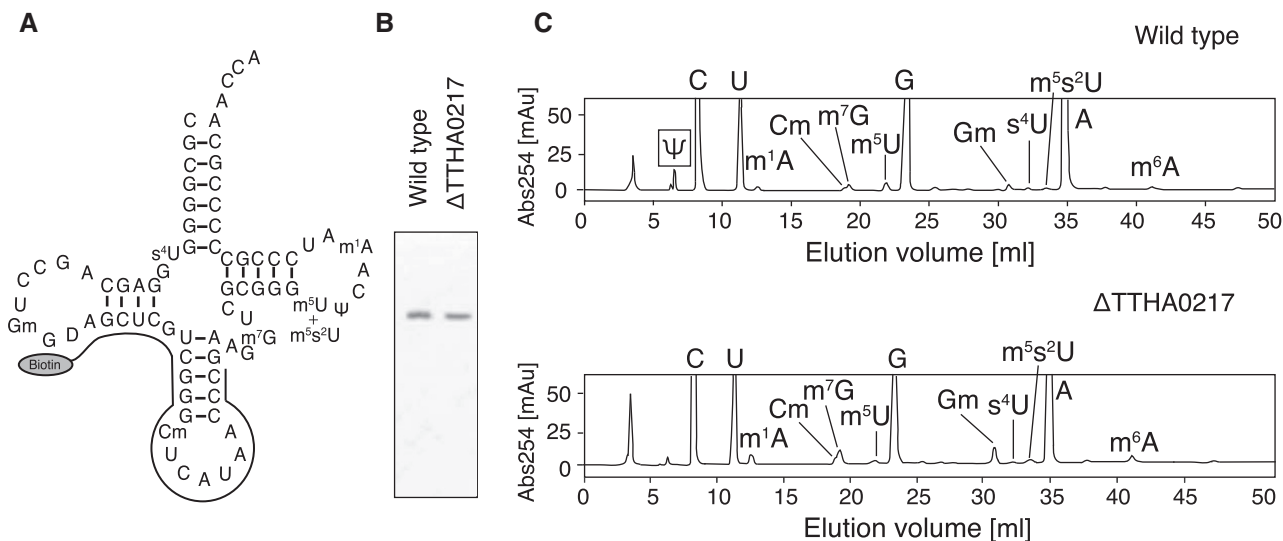


Figure 1. The $\Psi 55$ modification is absent in the purified tRNA^{Metf1} from the $\Delta TTHA0217$ strain. (A) Cloverleaf structure of *T. thermophilus* tRNA^{Metf1}. This tRNA has a single Ψ modification at position 55. The complimentary region to the DNA probe is illustrated. (B) Purified tRNA^{Metf1} molecules from the wild-type and $\Delta TTHA0217$ strains were analyzed by 10% PAGE (7 M urea). The gel was stained with toluidine blue. (C) The modified nucleosides in tRNA^{Metf1} from the wild type (upper) and the $\Delta TTHA0217$ (lower) cells cultured at 50°C were compared.

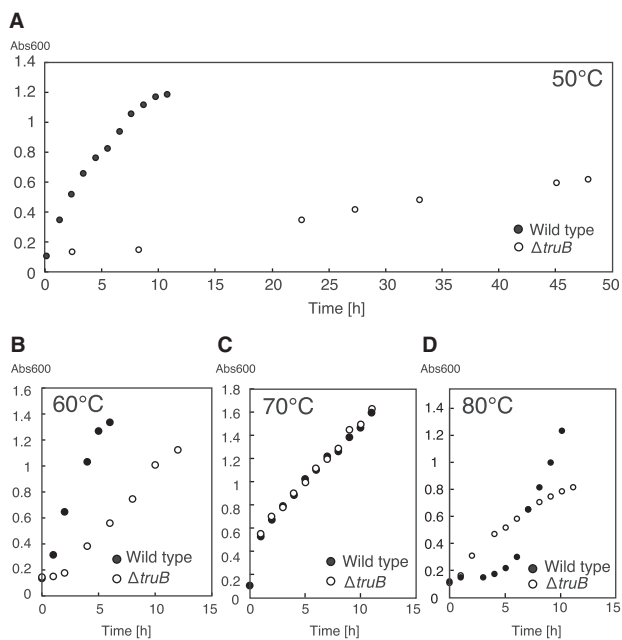


Figure 2. Growth phenotypes of the wild-type and $\Delta truB$ strains. The growth curves of the wild-type and $\Delta truB$ strains in liquid cultures at 50°C (A), 60°C (B), 70°C (C) and 80°C (D) are shown.

cultures at 50°C, 60°C, 70°C and 80°C. The wild type strain can live at a wide range of temperature (50–80°C). In contrast and to our surprise, severe growth retardation of the $\Delta truB$ strain was observed at 50°C (Figure 2A). At 60°C, the growth retardation was mitigated (Figure 2B). At 70°C, the wild-type and $\Delta truB$ strains exhibited similar growth (Figure 2C). At 80°C, a curious growth phenotype of the $\Delta truB$ strain was observed: the $\Delta truB$ strain did not have a lag-phase and showed an immediate exponential growth, which was slower than that of the wild-type strain

(Figure 2D). It should be mentioned that the pre-cultures were performed at 70°C. We repeated the experiments and confirmed that growth phenotypes were reproducibly observed at all temperatures. In general, the $\Psi 55$ modification contributes to stabilization of the L-shaped tRNA structure. Therefore, growth retardation at 80°C seemed to be reasonable although we could not explain the reason why the $\Delta truB$ strain did not show a lag-phase like the wild-type strain. We focused on unexpected growth retardation at low temperatures (<60°C). At low temperatures, tRNA structure is more stable compared to that at high temperatures. Furthermore, in general, lack of single modification in the three-dimensional core of tRNA does not cause any effect on cell growth at low temperatures (64). Even in the case of *T. thermophilus*, only three modifications [m^7G46 (60), m^5s^2U54 (65) and m^1A58 (63)] have been reported to affect cell growth and then only at high temperatures (especially 80°C). In the recent study, we reported that hypo-modifications in tRNA at high temperatures caused degradation of some tRNA species (60). However tRNA degradation only at low temperatures could not be assumed. Thus, this unexpected growth retardation at low temperatures is not explainable by loss of structural stability of tRNA through lack of the $\Psi 55$ modification.

Construction of *T. thermophilus* strains, which express mutant TruB proteins, for detection of RNA chaperone activity

Initially, we considered that lack of the RNA chaperone activity of TruB might cause the growth retardation at low temperatures if the RNA chaperone activity was genuine. The RNA chaperone activity of TruB protein in assisting the correct folding of tRNA was proposed by a competitive growth experiment with *E. coli* $\Delta truB$ strain and the wild-type strain (37). We considered that the RNA

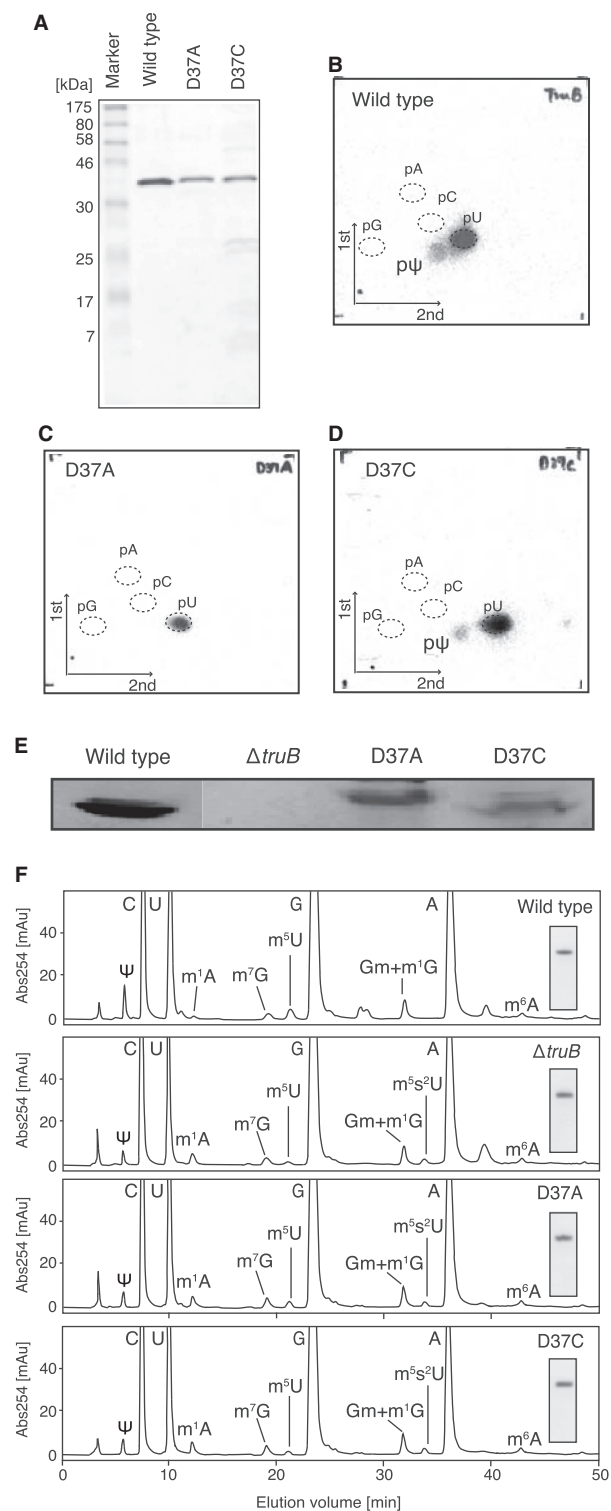


Figure 3. Enzymatic activities of the TruB wild-type, D37C and D37A proteins. (A) The TruB wild-type, D37A and D37C proteins were purified to near-homogeneity on 15% SDS-PAGE analysis. The gel was stained with Coomassie brilliant blue. The Ψ55 formation activities of the wild-type (B), D37A (C) and D37C (D) proteins were checked by 2D-TLC using ^{32}P -internal labeled tRNA^{Thr} transcript as described in the text. (E) The presence of the TruB wild-type, D37A and D37C proteins in the cell extracts were checked by western blot analysis. In contrast, the band corresponding to TruB was absent in the ΔtruB strain sample. (F) Nucleosides in 0.2 A₂₆₀ units of class I tRNA fractions from the wild-type and ΔtruB cells cultured at 60°C were analyzed

chaperone activity of TruB might be required only at low temperatures in the case of *T. thermophilus* because tRNA has structural softness (flexibility) at high temperatures. To verify this idea, we constructed *T. thermophilus* strains, which express mutant TruB proteins. Fortunately, the catalytic mechanism of TruB has been well studied (28–36) and the catalytic center has been verified (33,34). Based on amino acid sequence alignment (Supplementary Figure S1), we expected the aspartic acid at position 37 (D37), which corresponds to D48 in *E. coli* TruB, to be the catalytic center. We substituted D37 by alanine and cysteine individually because it was reported that these substitutions of the catalytic center produced severe loss of enzymatic activity (36). We purified recombinant D37A and D37C mutant proteins which were expressed in *E. coli* cells (Figure 3A). Ψ formation activities were checked by 2D-TLC. Briefly, *T. thermophilus* tRNA^{Thr} (GGT) was transcribed with T7 RNA polymerase and α - ^{32}P -UTP. The transcript was treated with the TruB wild-type, D37A, or D37C protein at 55°C for 1 h and then digested with nuclease P1 completely. The resultant nucleotides were separated on 2D-TLC and analyzed by autoradiography. As shown in Figure 3B, ^{32}P -pΨ was clearly observed in the wild-type sample. In contrast, ^{32}P -pΨ was not detected in the D37A sample (Figure 3C), demonstrating a near-completely loss of activity in the D37A mutant protein. Furthermore, a faint spot of ^{32}P -pΨ was observed in the D37C sample (Figure 3D), showing that the D37C mutant protein has very weak Ψ formation activity as in the case of *E. coli* TruB (36). It should be mentioned that Figure 3 does not represent the relative initial velocities: in order to demonstrate the weak activity of the D37C mutant enzyme, incubation was prolonged for 1 h. The activity of the D37C mutant enzyme is ~1% of that of the wild-type enzyme (data not shown). Thus, we successfully prepared mutant *truB* genes. We constructed the gene replacement vectors as shown in Supplementary Figure S3 and performed homologous recombination. *Thermus thermophilus* mutant strains were isolated and their DNA sequences were checked (data not shown). Expression of the mutant TruB proteins in *T. thermophilus* cells was confirmed by western blotting analysis (Figure 3E). The TruB mutant proteins as well as the wild-type protein were clearly detected in the S-100 fractions. In contrast, the band corresponding to the TruB protein is absent in the ΔtruB strain sample. Thus, we confirmed the expression of TruB D37A and D37C proteins in the *T. thermophilus* mutant strains. Ψ contents in tRNA from the wild-type, ΔtruB , D37A and D37C strains were checked by the HPLC nucleoside analysis of class I tRNA fractions (Figure 3F) because removal of rRNA fragments from total tRNA fraction was difficult. The wild-type, ΔtruB , D37A and D37C strains were cultured at 60°C. Total tRNA fraction from

by HPLC C18 reverse phase column chromatography. The class I tRNA fractions were analyzed by 10% PAGE (7 M urea) (Insets). The Ψ nucleoside was eluted at 6.7 ml. Elution points of the labeled nucleosides were confirmed by standard markers or enzyme formation as described in our recent report (60).

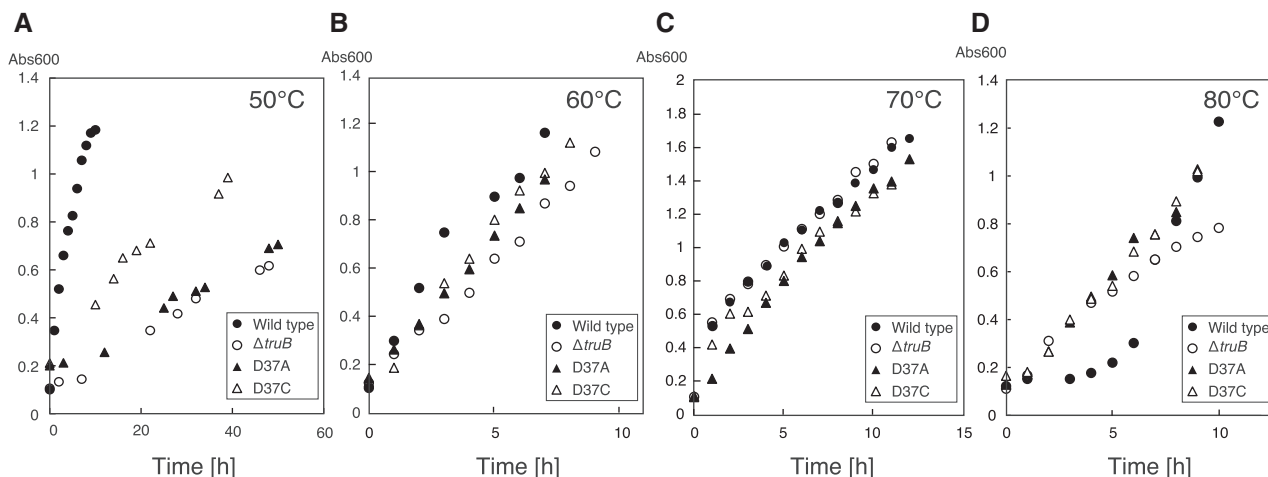


Figure 4. Effect of the TruB mutant proteins on cell growth was tested. The growth curves of the wild-type, *ΔtruB*, D37A and D37C strains were compared at 50°C (A), 60°C (B), 70°C (C) and 80°C (D).

each strain was prepared by phenol extraction and then the class I tRNA fraction was excised by 10% PAGE (7M urea) as shown in the insets of Figure 3F. Each class I tRNA was then completely digested with phosphodiesterase, bacterial alkaline phosphatase and RNase A. In the class I tRNA from the wild-type strain, Ψ was clearly observed because five types of tRNA Ψ synthases (TruA, TruB, RsuA, RluA and TruD) probably exist in the cells. In contrast, in the class I tRNA from the *ΔtruB* strain, the content of Ψ was dramatically decreased, consistent with deletion of *truB*. A similar result was obtained from the D37A mutant strain sample, suggesting that Ψ 55 in tRNA is absent even though mutant TruB D37A protein is expressed. Furthermore, Ψ content in the class I tRNA from the D37C mutant strain was slightly increased relative to the D37A mutant, consistent with the weak Ψ 55 formation activity of TruB D37C mutant protein. Thus, we confirmed that *T. thermophilus* TruB D37A and D37C mutant strains were successfully constructed.

RNA chaperone activity of TruB protein might exist, but the growth retardation at low temperatures could not be explainable by the RNA chaperone activity

In order to clarify whether the RNA chaperone activity of TruB really exists and has positive effects on cell growth, we investigated the growth phenotype of the wild-type, *ΔtruB*, D37A and D37C strains at 50°C (Figure 4A). To our disappointment, the growth of the D37A strain was not perfectly recovered but was slightly faster than that of the *ΔtruB* strain. In contrast, the growth of the D37C strain was obviously faster than that of the D37A and *ΔtruB* strains. Furthermore, the wild-type strain grew faster than any mutant strain. Thus, these experimental results revealed that the growth retardation of the *ΔtruB* strain at 50°C was mainly caused by lack of the Ψ 55 modification in tRNA although our experimental results did not contradict the RNA chaperone hypothesis. Thus, the growth retardation was not explainable by the RNA chaperone activity. We further analyzed the growth phenotypes

at 60°C, 70°C and 80°C (Figure 4B–D). At 60°C, recovery of growth by expression of the D37A and D37C mutant proteins was still observed however the effect was weaker than that at 50°C (Figure 4B). At 70°C, all strains showed similar growth (Figure 4C). At 80°C, unexpectedly, both D37A and D37C mutant strains grew considerably faster than the *ΔtruB* strain without the presence of a lag-phase. These phenomena might be caused by the RNA chaperone activity, which functions in exponential growth at high temperatures.

Abnormal enhancement of other tRNA modifications in the *ΔtruB* strain at 50°C

During the course of the study, we investigated the *T. thermophilus trmB* disruption strain (60). The gene, *trmB* encodes tRNA (m^7G46) methyltransferase (60,62,66). In a previous study, we found that the lack of m^7G46 modification causes hypo-modifications of other modified nucleotides such as Gm18 and m^1G37 (60). Thus, m^7G46 modification has positive effects on other modifications, suggesting that there is a tRNA modification network(s). We considered that Ψ 55 might have effects on other tRNA modifications. To confirm this idea, we performed nucleoside analysis of class I tRNA from the wild-type and *ΔtruB* strains cultured at 50°C and 70°C. The class I tRNA fractions were prepared as shown in Figure 5A. Nucleosides in these class I tRNA fractions were analyzed (Figure 5B and C). Surprisingly, the amount of several modified nucleotides such as m^1A , Gm + m^1G and m^5s^2U increased in the *ΔtruB* strain cultured at 50°C as compared to that in the wild-type sample (Figure 5B). It is known that the amount of these modifications (m^1A , Gm and m^5s^2U) in *T. thermophilus* tRNA increase with culture temperature (58,60,67). In fact, these modifications in the class I tRNA from the wild-type strain cultured at 70°C clearly increased as compared to those at 50°C (Figure 5B and C, upper panels). However these nucleosides in the class I tRNA of the *ΔtruB* strain cultured at 50°C were abnormally increased (Figure 5B, lower panel).

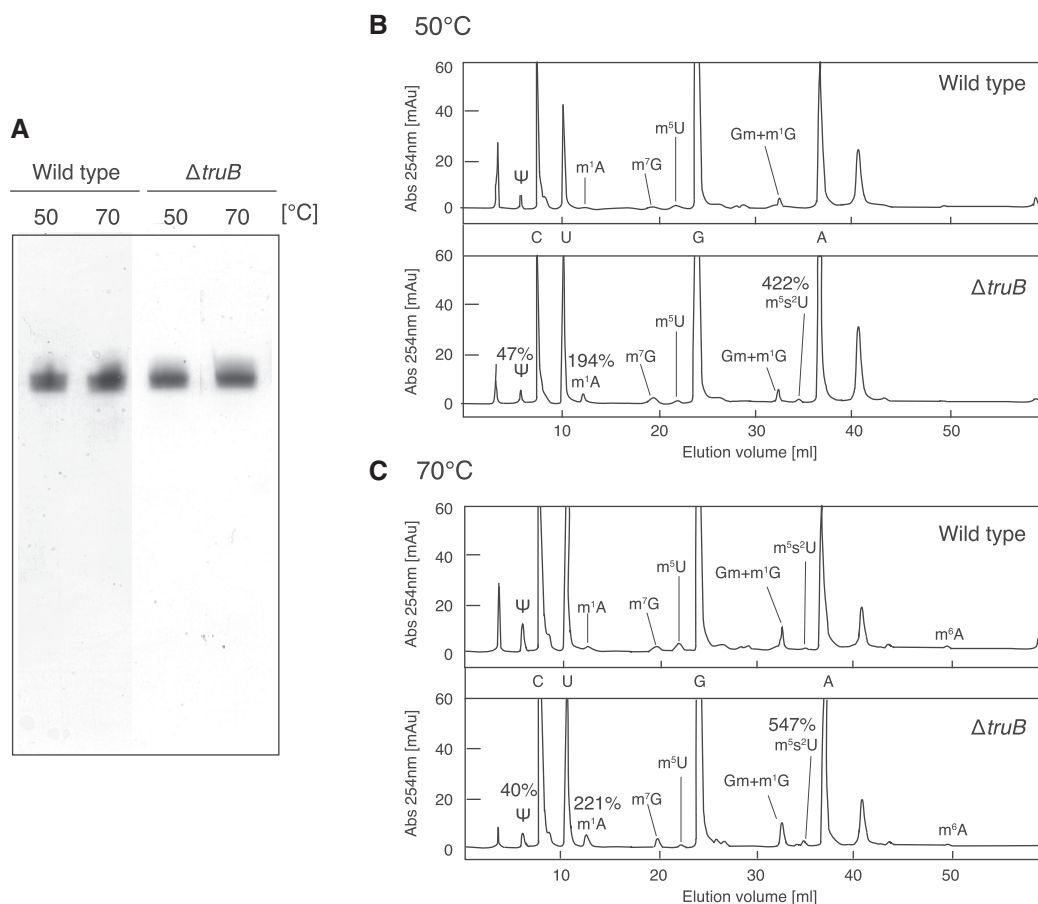


Figure 5. Nucleoside analysis of class I tRNA fractions from the wild-type and $\Delta truB$ cells cultured at 50°C and 70°C. (A) The class I tRNA fractions (0.05 A260 units) were analyzed by 10% PAGE (7M urea). The gel was stained with toluidine blue. The modified nucleosides in the class I tRNA fractions from the cells cultured at 50°C (B) and 70°C (C) were analyzed by HPLC C18 reverse phase column chromatography. The ratio of modified nucleoside was calculated from the peak area: the peak area in the wild-type sample at 50°C was expressed as 100%.

Modification assay for tRNA methyltransferases

In order to estimate the modified nucleoside contents in tRNA correctly, we prepared four tRNA modification enzymes, TrmH, TrmB, TrmA and TrmI (Figure 6A). TrmH, TrmB, TrmA and TrmI are tRNA (Gm18) methyltransferase (40–42), tRNA (m⁷G46) methyltransferase (60,62,66), AdoMet-dependent tRNA (m⁵U54) methyltransferase (47–49) and tRNA (m¹A58) methyltransferase (63), respectively. It should be mentioned that the m⁵U54 modification in *T. thermophilus* is generated by the N⁵, N¹⁰-methylene tetrahydrofolate-dependent enzyme (TrmFO) (50,51). However N⁵, N¹⁰-methylene tetrahydrofolate is unstable and radioisotope labeled compound is not commercially available. Therefore we used *E. coli* TrmA instead of TrmFO. The tRNA mixtures from the wild-type and $\Delta truB$ strains cultured at 50°C were methylated with ¹⁴C-AdoMet by addition of these individual enzymes for 1 h, 3 h and over-night. Because the ¹⁴C-methyl group incorporation was not changed for 3 h and over-night incubations, we judged that the tRNA mixtures were near-completely methylated (Figure 6B). As shown in Figure 6B, the Gm18 and m¹A58 content in the wild-type sample was clearly lower than that seen in the $\Delta truB$ strain sample,

consistent with the HPLC nucleoside analysis. These results suggest that the Ψ 55 modification has a negative effect on the TrmH and TrmI reactions. The m⁷G46 modification was near-completed in both the wild-type and $\Delta truB$ strain samples although the content in the $\Delta truB$ strain was slightly more than that in the wild-type sample, suggesting that the presence of the Ψ 55 modification does not affect the TrmB reaction significantly. To our surprise, a considerable amount of U54 in both the wild-type and $\Delta truB$ strains remained as unmodified U54 (or s²U54), demonstrating that TrmFO works effectively at high temperatures. Thus, we confirmed that the Ψ 55 modification does not significantly affect methylation of U54. It should be mentioned that nucleoside analysis in Figure 5B clearly showed an increase of m⁵s²U in the $\Delta truB$ strain cultured at 50°C. Taking these experimental results together, we conclude that the presence of the Ψ 55 modification has a negative effect on formation of the m⁵s²U54 from the m⁵U54 although the Ψ 55 modification does not have a significant effect on the m⁵U54 modification at least at 50°C. The sulfur-transfer reaction in m⁵s²U54 formation is very complicated as reported by Shigi with at least, four proteins (TtuA, TtuB, TtuC and IscS) being involved in the reaction (65,67,68). Therefore we could not investigate

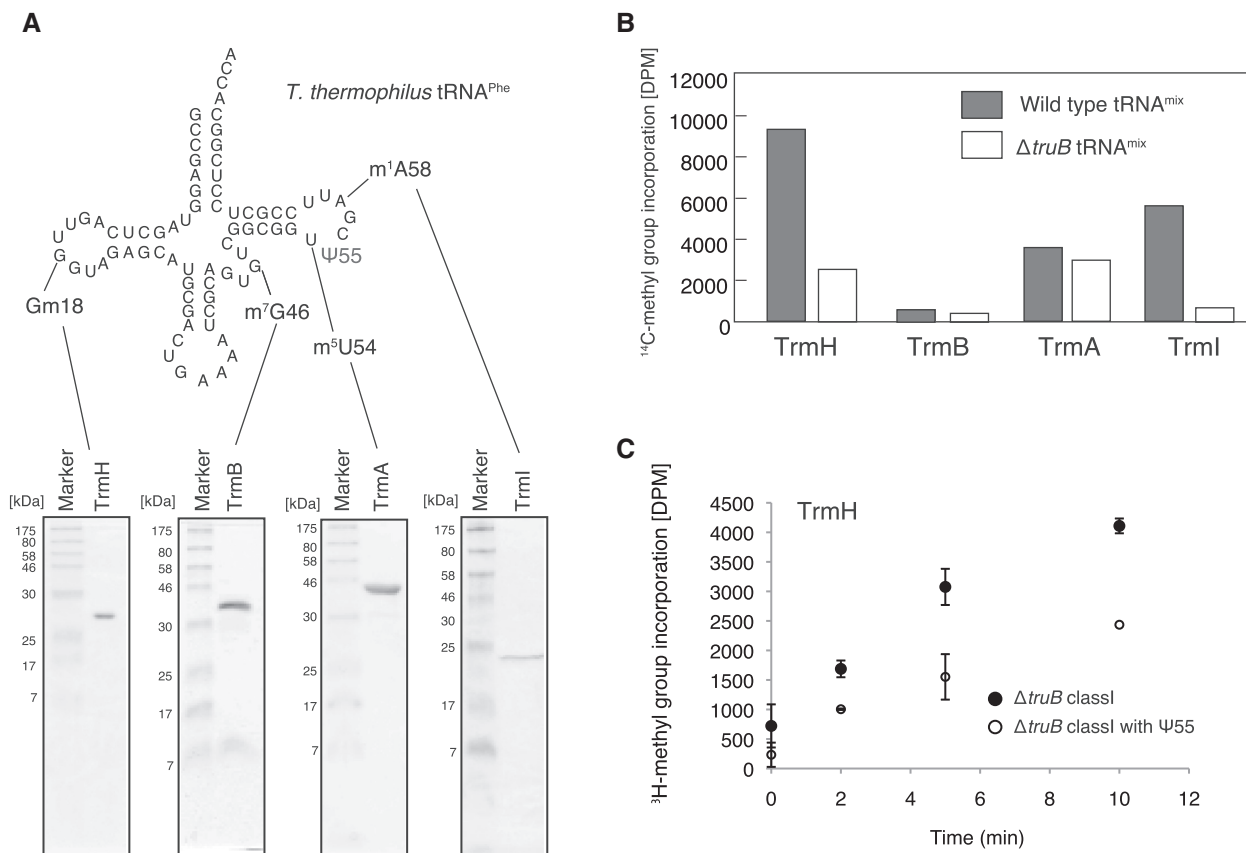


Figure 6. Modification assay with tRNA methyltransferases. (A) *T. thermophilus* tRNA^{Phe} is depicted in the cloverleaf structure. Modification sites (Gm18, m⁷G46, m⁵U54 and m¹A58) are shown in the figure. The corresponding modification enzymes (TrmH, TrmB, TrmA and TrmI) on 15% SDS-PAGE are shown. Each gel was stained with Coomassie brilliant blue. (B) Mixtures of tRNA from the wild-type and Δ truB cells cultured at 50°C were prepared and then served as substrate for tRNA methyltransferases as described in ‘Materials and Methods’ section. (C) *Thermus thermophilus* class I tRNA from Δ truB strain were modified with TruB for 3 h at 55°C. Then methyl group acceptance activities with and without Ψ 55 modification in reactions catalyzed by TrmH were compared.

the effect of the Ψ 55 modification on the sulfur-transfer reaction enzymatically. However our current experimental results suggest two hypothetical schemes. One is that the Ψ 55 modification has a direct negative effect on these sulfur-transfer proteins (or complex). The other is that the Ψ 55 modification has a negative effect only on the m¹A58 formation and enhanced m¹A58 modification through a lack of the Ψ 55 modification inducing the sulfur transfer reaction since the m¹A58 modification has been reported to be a positive determinant for the sulfur-transfer reaction (67).

Next, we performed a time-dependent assay with unmodified and Ψ 55 modified class I tRNA from the Δ truB strain. The class I tRNA from the Δ truB strain was modified with TruB protein for 3 h and then used as the substrate for TrmH. As shown in Figure 6C, for TrmH, unmodified class I tRNA was modified faster than the class I tRNA with Ψ 55. Thus, *in vitro* TrmH reaction is consistent with the *in vivo* modification. The negative effect of the Ψ 55 modification on Gm18 modification is explainable without considering other protein (or RNA) factors. The presence of the Ψ 55 modification brings rigidity to the local structure of tRNA (5). This rigidity

probably decreases the speed of the induced fit process of TrmH at least at 55°C (46).

Abnormal high melting temperature of class I tRNA from the Δ truB strain

In the absence of the Ψ 55 modification, the amounts of Gm18, m¹A58 and m⁵s²U54 modifications were abnormally increased. In this case the melting temperature of tRNA from the Δ truB strain would be expected to be increased. We therefore analyzed the melting profiles of class I tRNAs from the wild-type and Δ truB strains cultured at 50°C and 70°C (Figure 7A and B). The melting temperature of tRNA from the Δ truB strain cultured at 50°C was much higher than that of the wild-type sample (Figure 7A and C). This abnormally high melting temperature suggests that the tRNA structure may be too rigid to work at 50°C, because the m⁵s²U54 modification in tRNA^{Phe} depresses the poly (U) dependent-poly (Phe) translational activity at 50°C in the case of *T. thermophilus* (69). In contrast, tRNAs from the wild-type and Δ truB strains cultured at 70°C showed similar melting profiles, consistent with the

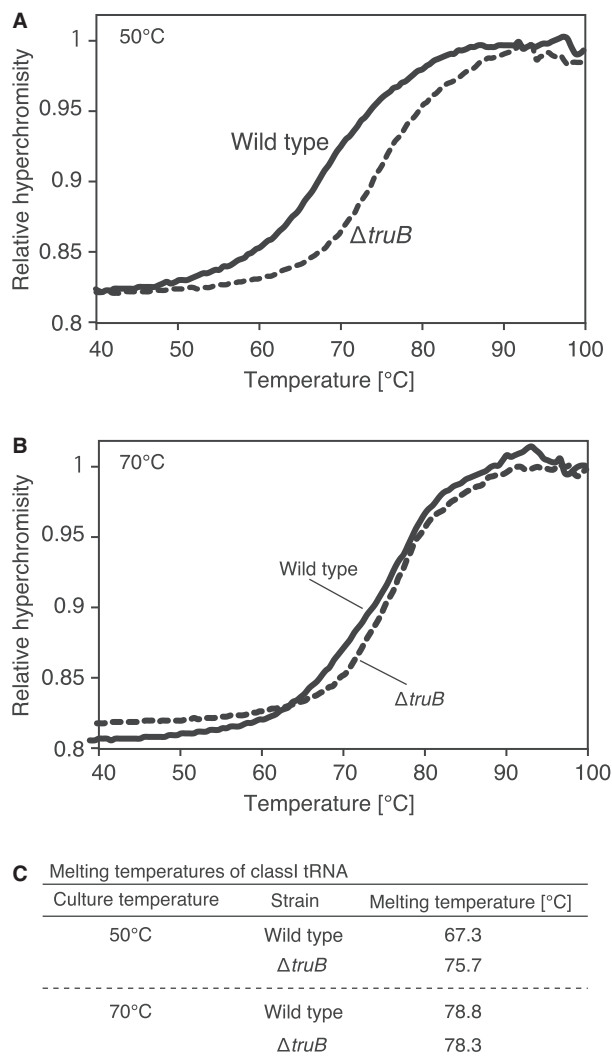


Figure 7. Melting profiles of the class I tRNA from the wild-type and $\Delta truB$ cells cultured at 50°C (A) and 70°C (B). The profiles >90°C were perturbed by bubbles generated in the samples with increased temperature. The calculated melting temperatures are summarized in (C).

growth phenotypes (Figure 2C) and nucleoside analysis (Figure 5A).

^{35}S -Met incorporation of the $\Delta truB$ strain is inferior to that of the wild-type strain at 50°C and 70°C

In order to verify whether the protein synthesis activity of the $\Delta truB$ strain is depressed at 50°C, we investigated the ^{35}S -Met incorporation (Figure 8). The wild-type and $\Delta truB$ strains were cultured at 50°C or 70°C. When the cell density ($A_{600\text{nm}}$) had reached 0.6, ^{35}S -Met was directly supplemented into the medium. As shown in Figure 8, ^{35}S -Met incorporation into the wild-type cells was clearly observed at both 50°C and 70°C. In contrast, ^{35}S -Met incorporation of the $\Delta truB$ strain was hardly detectable at 50°C. Although ^{35}S -Met incorporation of the $\Delta truB$ strain was observed at 70°C, it was considerably inferior to that of the wild-type strain. Thus, these results clearly showed that the protein synthesis activity

of the $\Delta truB$ strain is inferior to that of the wild-type strain at both 50°C and 70°C.

These experiments brought us additional information. In the case of the wild-type strain, synthesized protein species were different at 50°C and 70°C (Figure 8). At 50°C, small proteins were abundantly synthesized. In fact, accumulation of the small proteins at 50°C was observed in the wild-type sample (Figure 8, upper panel). These bands were corresponding to the sizes of the cold-shock proteins in *T. thermophilus*. *Thermus thermophilus* has at least two cold-shock proteins (ttCsp1 and ttCsp2) (70). The expected sizes of ttCsp1 (TTHA0175) and ttCsp2 (TTHA0359) are 8184 and 7768 Da, respectively. In contrast, these bands were not observed in the $\Delta truB$ strain sample. Thus, the depression of the protein synthesis activity in the $\Delta truB$ strain causes deficit of the cold-shock proteins at 50°C. Probably, the deficit of the cold-shock proteins blunts the cell responses in metabolism, transcription, translation and protein folding to low temperatures (70) and causes severe growth defect at 50°C.

DISCUSSION

In this study, we investigated a *truB* gene disruptant mutant of *T. thermophilus*, an extreme-thermophilic eubacterium. Unexpectedly, the $\Delta truB$ strain exhibited severe growth retardation at low temperatures (especially 50°C). Some tRNA modifications such as Gm18, $m^5s^2\text{U}54$ and $m^1\text{A}58$ were abnormally enhanced in class I tRNA from the $\Delta truB$ strain cultured at 50°C. These enhanced modifications produce an unwanted increase in the melting temperature of tRNA. In fact, the protein synthesis activity of the $\Delta truB$ strain was clearly inferior to that of the wild-type strain at both 50°C and 70°C. Furthermore, the bands corresponding to the cold-shock proteins were absence in the $\Delta truB$ strain sample cultured at 50°C. This deficit of the cold-shock proteins in the $\Delta truB$ cells probably causes the severe growth defect at 50°C. At least at 50°C, the $\Psi55$ modification controls the content of other modifications in tRNA. This idea was confirmed by nucleoside analysis of modified tRNA *in vivo* and in an *in vitro* modification assay with tRNA modification enzymes. Thus, the $\Psi55$ modification is required for low-temperature adaptation for the organism. For a long time, modified nucleotides in the 3D-core of tRNA have been mainly considered as tRNA stabilization factors. However our current study provides a new concept, namely that a decrease of amounts of modified nucleotides in the 3D-core of tRNA brings structural softness (flexibility) to tRNA in some cases (for example, at low temperatures for *T. thermophilus*) and functions as an environment adaptation factor. In this system, the $\Psi55$ modification controls the tRNA modification network.

Based on our previous (60) and current studies and research by others (63,67), the network of modified nucleotides and tRNA modification enzymes in *T. thermophilus* can be drawn as shown in Figure 9. It should be mentioned that each modification enzyme can

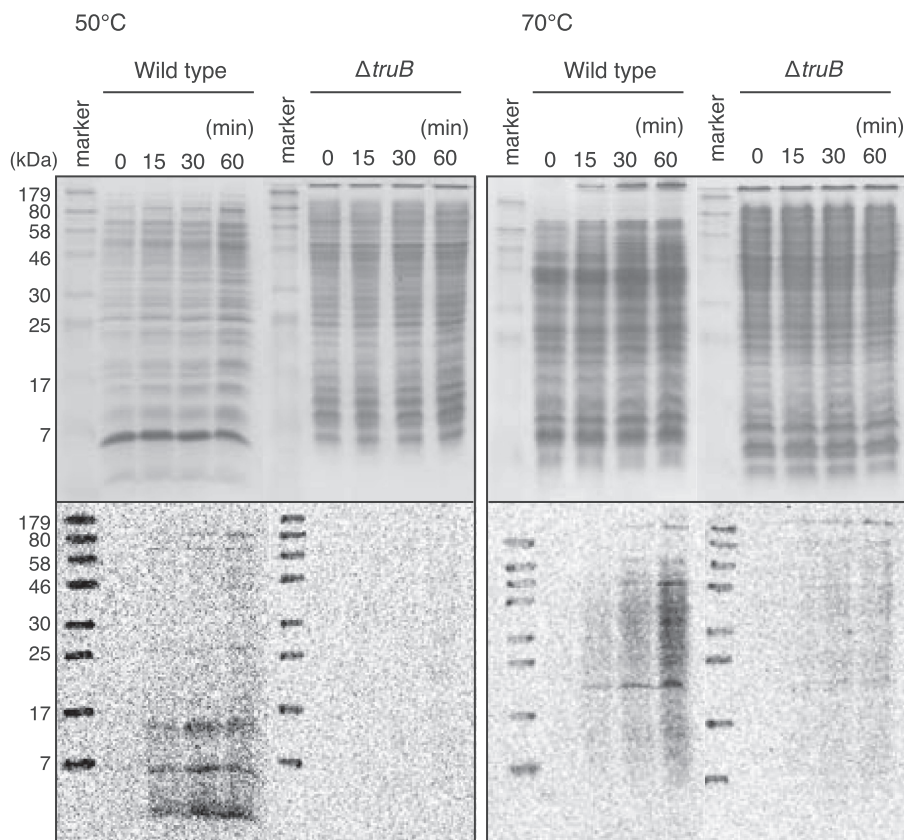


Figure 8. Translation activity analysis by ^{35}S -Met incorporation. The wild-type and ΔtruB cells were cultured at 50°C (left) and 70°C (right). At 0 min point, ^{35}S -Met was supplemented into the medium. The cells were collected at 0, 15, 30 and 60 min. The proteins were analyzed by 15% SDS-PAGE (upper). The gel was stained with Coomassie brilliant blue. The lower panels show the autoradiogram of the gel.

act on unmodified tRNA transcript. This figure shows positive and negative effects of the modified nucleotides on other modification enzymes. Of course, the modified nucleotide itself acts as a competitive inhibitor of the corresponding modification enzyme. For example, increase of Gm18 content in tRNA inhibits TrmH activity (46). Furthermore, this network is observed in extreme thermophilic eubacterium *T. thermophilus*, which can grow at 50 – 80°C . In the network, two modified nucleotides, $\Psi 55$ and $\text{m}^7\text{G46}$ work as key factors. As described in this article, the presence of the $\Psi 55$ modification has a negative effect on TrmH activity *in vivo* and *in vitro*. Although sulfur-transfer to $\text{m}^5\text{U54}$ increases in the ΔtruB strain cells cultured at 50°C , this phenomenon is not confirmed by *in vitro* assay. Therefore, this may be indirectly induced by increase in $\text{m}^1\text{A58}$ content (63,67). The negative effect of the $\Psi 55$ modification on $\text{m}^1\text{A58}$ modification was confirmed *in vivo*. To clarify the precise modification mechanism by TrmI, further study will be necessary. In contrast, the presence of $\text{m}^7\text{G46}$ modification accelerates modification velocities of Gm18 by TrmH and $\text{m}^1\text{G37}$ by TrmD *in vivo* and *in vitro* (60). The positive effect of the $\text{m}^7\text{G46}$ modification on TrmI activity was confirmed only *in vitro*, suggesting that a sufficient amount of the TrmI protein may exist in the *T. thermophilus* cells (60). In the network, the $\text{m}^5\text{s}^2\text{U54}$ modification mainly contributes to increase of melting temperature (5,58,67).

Although all protein factors for the $\text{m}^5\text{s}^2\text{U54}$ modification have not been identified (65,68), understanding of the $\text{m}^5\text{s}^2\text{U54}$ formation pathway will be necessary to elucidate the all of the temperature-dependent tRNA adaptation system of thermophiles. In Figure 9, not all common modifications in the 3D-core are depicted. For example, D20 modifications in tRNA^{Met} and tRNA^{Phe} from *T. thermophilus* have been reported (2). In the case of *E. coli*, the D modifications in tRNA are produced by three dihydrouridine synthases (Dus) (71) however only one gene could be found in *T. thermophilus* genome. Therefore, experimental identification of the dihydrouridine synthase gene in *T. thermophilus* is necessary. In general, the D modification confers flexibility to the local structure of RNA (72). Furthermore, in the case of yeast, the Gm18 modification by Trm3 is required for the D20 modification by Dus2 (73–75). In the future study, role of the D modification(s) should be investigated in *T. thermophilus* tRNA modification network.

In the current study, we found that expression of D37A mutant protein clearly produced recovery of the growth speed at 80°C (Figure 4D). At 50°C , a slight recovery in the growth speed was also observed (Figure 4A). Because we constructed the mutant strain by homologous recombination, the mutant TruB gene existed as only one copy in the genome. If we supplied the mutant protein into the cell by plasmid vectors, the recovery at 50°C would be

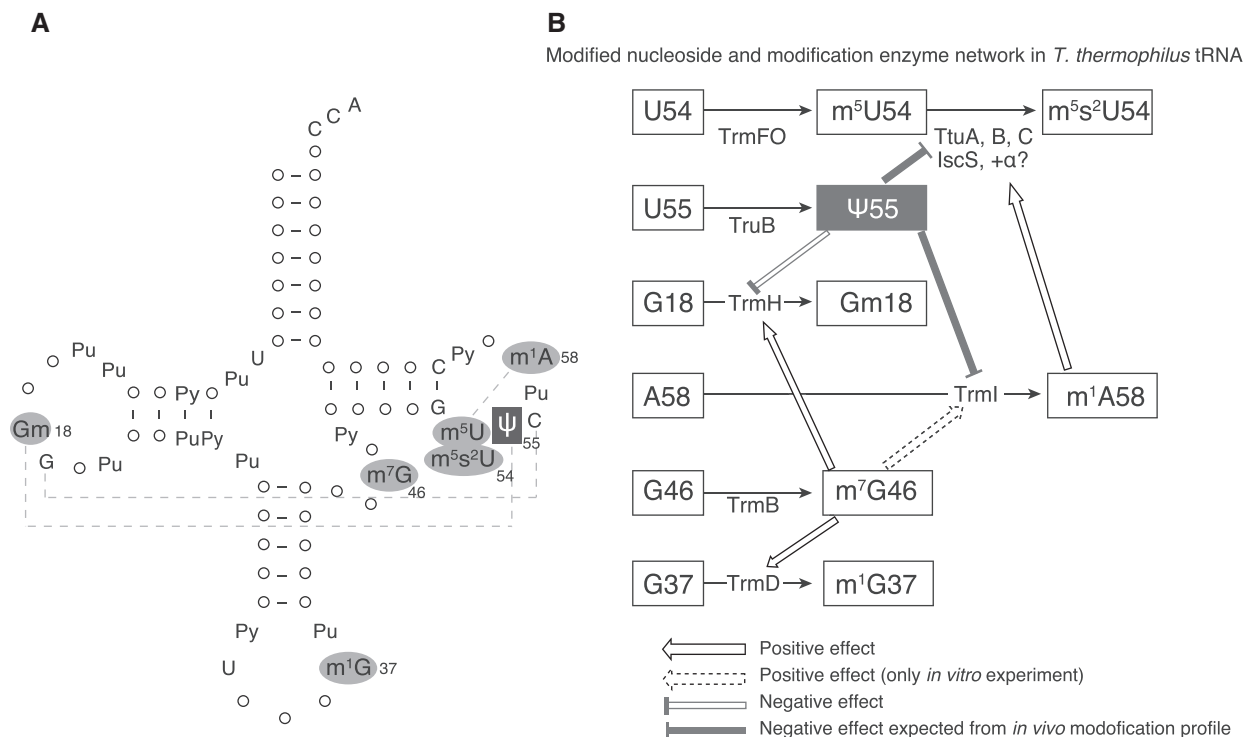


Figure 9. Modified nucleotide and modification enzyme network in *T. thermophilus* tRNA. (A) The modification positions are depicted in the cloverleaf structure of typical tRNA. The conserved nucleotides are shown by A, U, G and C. Py and Pu are abbreviations for pyrimidine and purine, respectively. Broken lines are tertiary base pairs. Numbers show positions of the modifications. (B) This figure shows the positive or negative effect of modified nucleotide on other modifications. Nucleotides and positions are boxed. The modification enzyme which catalyzes each reaction is shown for each pathway. This network maintains an adequate balance of tRNA modifications and responds to environmental changes (for example, culture temperature).

expected to be enhanced. Our experimental results do not contradict the hypothesis by Gutgsell *et al.* (37). However there are two other possibilities. One is that the TruB protein binds to unidentified protein(s) and this complex has another function at 80°C. Because TruB protein itself may affect other proteins on the precursor tRNA, we cannot neglect this possibility. The other is that TruB protein binds to tRNA and protects from cleavages by nucleases. Although the amount of tRNA is much than that of the TruB, this protection should be considered. If the TruB has the RNA chaperone activity, it suggests one idea: other 3D-core modification enzymes may have a RNA chaperone activity. To clarify the idea, further studies will be necessary. The D37A and D37C mutants as well as the Δ *truB* strain do not show a lag-phase in the culture at 80°C. This fact suggests that the Ψ 55 modification works at a lag-phase in the modified nucleotide and modification enzyme network. Majority of the basic modifications in tRNA seemed to be taking place in a lag-phase.

The Ψ 55 modification was found in *A. aeolicus* tRNA^{Cys} (59) and *T. maritima* has a TruB protein (27,28). Furthermore *truB* genes are found in almost all eubacterial genomes (23,24). These facts suggest that the Ψ 55 modification and TruB protein existed in the common ancestor of eubacteria. Because the common ancestor probably lived in a high-temperature environment, the Ψ 55 modification probably functioned on tRNA

stabilization. According to cooling of the Earth, thermophiles should adapt to low temperatures for survival. During this process, the Ψ 55 modification might have obtained a new function, which controls other modifications. In mesophiles, the Ψ 55 modification may have been changed to a stress resistance factor (25,26,37,39).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Dunin-Horkawicz, S., Czerwoniec, A., Gajda, M.J., Feder, M., Grosjean, H. and Bujnicki, J.M. (2006) MODOMICS: a database of RNA modification pathways. *Nucleic Acids Res.*, **34**, D145–149.
- Rozanski, J., Crain, P.F. and McCloskey, J.A. (1999) The RNA Modification Database: 1999 update. *Nucleic Acids Res.*, **27**, 196–197.
- Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A. and Steinberg, S. (1998) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.*, **26**, 148–153.
- Ofengand, J. and Fournier, M.J. (1998) The pseudouridine residues of tRNA: number, location, biosynthesis and function. In Grosjean, H. and Benne, R. (eds), *Modification and Editing of RNA*. American Society for Microbiology, Washington, DC, pp. 229–253.
- Motorin, Y. and Helm, M. (2010) tRNA stabilization by modified nucleotides. *Biochemistry*, **49**, 4934–4944.
- Motorin, Y., Muller, S., Behm-Ansmant, I. and Branlant, C. (2007) Chapter 2. 4. 2. Detection of pseudouridine (Ψ) residues. In Gott, J.M. (ed.), *Methods in Enzymol.*, Vol. 245. Elsevier, San Diego, California, USA, pp. 36–41.
- Garcia, G.A. and Goodenough-Lashua, D.M. (1998) Appendix 3: General properties of RNA-modifying and -editing enzymes. In Grosjean, H. and Benne, R. (eds), *Modification and Editing of RNA*. American Society for Microbiology, Washington, DC, pp. 555–560.
- Kim, S.H., Sussman, J.L., Suddath, F.L., Quigley, G.J., McPherson, A., Wang, A.H., Seeman, N.C. and Rich, A. (1974) The general structure of transfer RNA molecules. *Proc. Natl Acad. Sci. USA*, **71**, 4970–4974.
- Robertus, J.D., Ladner, J.E., Finch, J.T., Rhodes, D., Brown, R.S., Clark, B.F. and Klug, A. (1974) Structure of yeast phenylalanine tRNA at 3 Å resolution. *Nature*, **250**, 546–551.
- Johnson, L. and Söll, D. (1970) *In vitro* biosynthesis of pseudouridine at the polynucleotide level by an enzyme extract from *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **67**, 943–950.
- Schaefer, K.P., Altman, S. and Söll, D. (1973) Nucleotide modification *in vitro* of the precursor of transfer RNA^{Tyr} of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **70**, 3626–3630.
- Cortese, R., Kammen, H.O., Spengler, S.J. and Ames, B.N. (1974) Biosynthesis of pseudouridine in transfer ribonucleic acid. *J. Biol. Chem.*, **249**, 1103–1108.
- Arena, F., Ciliberto, G., Ciampi, S. and Cortese, R. (1978) Purification of pseudouridylate synthase I from *Salmonella typhimurium*. *Nucleic Acids Res.*, **5**, 4523–4536.
- Hur, S. and Stroud, R.M. (2007) How U38, 39, and 40 of many tRNAs become the targets for pseudouridylation by TruA. *Mol. Cell.*, **26**, 189–203.
- Del Campo, M., Kaya, Y. and Ofengand, J. (2001) Identification and site of action of the remaining four putative pseudouridine synthases in *Escherichia coli*. *RNA*, **7**, 1603–1615.
- Kaya, Y. and Ofengand, J. (2003) A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA*, **9**, 711–721.
- Nurse, K., Wrzesinski, J., Bakin, A., Lane, B.G. and Ofengand, J. (1995) Purification, cloning, and properties of the tRNA Ψ 55 synthase from *Escherichia coli*. *RNA*, **1**, 102–112.
- Roovers, M., Hale, C., Tricot, C., Terns, M.P., Terns, R.M., Grosjean, H. and Droogmans, L. (2006) Formation of the conserved pseudouridine at position 55 in archaeal tRNA. *Nucleic Acids Res.*, **34**, 4293–4301.
- Watanabe, Y.-I. and Gray, M.W. (2000) Evolutionary appearance of genes encoding proteins associated with box H/ACA snoRNAs: Cbf5p in *Euglena gracilis*, an early diverging eukaryote, and candidate Gar1p and Nop10p homologs in archaeobacteria. *Nucleic Acids Res.*, **28**, 2342–2352.
- Muller, S., Fourmann, J.-B., Loegler, C., Charpentier, B. and Branlant, C. (2007) Identification of determinants in the protein partners aCBF5 and aNOP10 necessary for the tRNA: Ψ 55-synthase and RNA guided RNA: Ψ -synthase activity. *Nucleic Acids Res.*, **35**, 5610–5624.
- Gurha, P. and Gupta, R. (2008) Archaeal Pus10 proteins can produce both pseudouridine 54 and 55 in tRNA. *RNA*, **14**, 2521–2527.
- Becker, H.F., Motorin, Y., Planta, R.J. and Grosjean, H. (1997) The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of Ψ 55 in both mitochondrial and cytoplasmic tRNAs. *Nucleic Acids Res.*, **25**, 4493–4499.
- Koonin, E.V. (1996) Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTPdeaminases. *Nucleic Acids Res.*, **24**, 2411–2415.
- Gustafsson, C., Reid, R., Greene, P.J. and Santi, D.V. (1996) Identification of new RNA modifying enzymes by iterative genome search using known modifying enzymes as probes. *Nucleic Acids Res.*, **24**, 3756–3762.
- Urbanavicius, J., Durand, M.B. and Björk, G.R. (2002) Three modifications in the D and T arms of tRNA influence translation in *Escherichia coli* and expression of virulence genes in *Shigella flexneri*. *J. Bacteriol.*, **184**, 5348–5357.
- Saga, A.E., Vasil, A.I. and Vasil, M.L. (1997) Molecular characterization of mutants affected in the osmoprotectant-dependent induction of phospholipase C in *Pseudomonas aeruginosa* PAO1. *Mol. Microbiol.*, **23**, 43–56.
- Wouters, J., Tricot, C., Durbecq, V., Roovers, M., Stalon, V. and Droogmans, L. (2003) Preliminary X-ray crystallographic analysis of tRNA pseudouridine 55 synthase from the thermophilic eubacterium *Thermotoga maritima*. *Acta Crystallogr. D Biol. Crystallogr.*, **59**, 152–154.
- Phannachet, K., Elias, Y. and Huang, R.H. (2005) Dissecting the roles of a strictly conserved tyrosine in substrate recognition and catalysis by pseudouridine 55 synthase. *Biochemistry*, **44**, 15488–15494.
- Gu, X., Yu, M., Ivanetich, K.M. and Santi, D.V. (1998) Molecular recognition of tRNA pseudouridine 55 synthase. *Biochemistry*, **37**, 339–343.
- Hoang, C. and Ferre-D'Amare, A.R. (2001) Cocrystal structure of a tRNA Ψ 55 pseudouridine synthase: nucleotide flipping by an RNA-modifying enzyme. *Cell*, **107**, 929–939.
- Pan, H., Agarwalla, S., Moustakas, D.T., Finer-Moore, J. and Stroud, R.M. (2003) Structure of tRNA pseudouridine synthase TruB and its RNA complex: RNA recognition through a combination of rigid docking and induced fit. *Proc. Natl Acad. Sci. USA*, **100**, 12648–12653.
- Hoang, C., Hamilton, C.S., Mueller, E.G. and Ferre-D'Amare, A.R. (2005) Precursor complex structure of pseudouridine synthase TruB suggests coupling of active site perturbations to an RNA sequestering peripheral protein domain. *Protein Sci.*, **14**, 2201–2206.
- Huang, L., Pookanjanatavip, M., Gu, X. and Santi, D.V. (1998) A conserved aspartate of tRNA pseudouridine synthase is essential for activity and a probable nucleophilic catalyst. *Biochemistry*, **37**, 344–351.
- Ramamurthy, V., Swann, S.L., Paulson, J.L., Spedalieri, C.J. and Mueller, E.G. (1999) Critical aspartic acid residues in pseudouridine synthases. *J. Biol. Chem.*, **274**, 22225–22230.
- Spedalieri, C.J., Hamilton, C.S. and Mueller, E.G. (2000) Functional importance of motif I of pseudouridine synthases: mutagenesis of aligned lysine and proline residues. *Biochemistry*, **39**, 9459–9465.
- Hamilton, C.S., Spedalieri, C.J., Ginter, J.M., Johnston, M.V. and Mueller, E.G. (2005) The roles of the essential Asp-48 and highly conserved His-43 elucidated by the pH dependence of the pseudouridine synthase TruB. *Arch. Biochem. Biophys.*, **433**, 322–334.
- Gutgsell, N., Englund, N., Niu, L., Kaya, Y., Lane, B.G. and Ofengand, J. (2000) Deletion of the *Escherichia coli* pseudouridine synthase gene *truB* blocks formation of pseudouridine 55 in

- tRNA *in vivo*, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells. *RNA*, **6**, 1870–1881.
38. Urbonavicius, J., Stahl, G., Durand, J.M.B., Ben Salem, S.N., Qian, Q., Farbaugh, P.J. and Björk, G.R. (2003) Transfer RNA modifications that alter +1 frameshifting in general fail to affect -1 frameshifting. *RNA*, **9**, 760–768.
 39. Kinghorn, S.M., O'Byrne, C.P., Booth, I.R. and Stansfield, I. (2002) Physiological analysis of the role of *truB* in *Escherichia coli*: a role for tRNA modification in extreme temperature resistance. *Microbiology*, **148**, 3511–3520.
 40. Persson, B.C., Jager, G. and Gustafsson, C. (1997) The *spoU* gene of *Escherichia coli*, the fourth gene of the *spoT* operon, is essential for tRNA (Gm18) 2'-O-methyltransferase activity. *Nucleic Acids Res.*, **25**, 4093–4097.
 41. Hori, H., Suzuki, T., Sugawara, K., Inoue, Y., Shibata, T., Kuramitsu, S., Yokoyama, S., Oshima, T. and Watanabe, K. (2002) Identification and characterization of tRNA (Gm18) methyltransferase from *Thermus thermophilus* HB8: domain structure and conserved amino acid sequence motifs. *Genes Cells*, **7**, 259–272.
 42. Hori, H., Kubota, S., Watanabe, K., Kim, J.M., Ogasawara, T., Sawasaki, T. and Endo, Y. (2003) *Aquifex aeolicus* tRNA (Gm18) methyltransferase has unique substrate specificity. *J. Biol. Chem.*, **278**, 25081–25090.
 43. Nureki, O., Watanabe, K., Fukai, S., Ishii, R., Endo, Y., Hori, H. and Yokoyama, S. (2004) Deep knot structure for construction of active site and cofactor binding site of tRNA modification enzyme. *Structure*, **12**, 593–604.
 44. Watanabe, K., Nureki, O., Fuukai, S., Ishii, R., Okamoto, H., Yokoyama, S., Endo, Y. and Hori, H. (2005) Roles of conserved amino acid sequence motifs in the SpoU (TrmH) RNA methyltransferase family. *J. Biol. Chem.*, **280**, 10368–10377.
 45. Watanabe, K., Nureki, O., Fukai, S., Endo, Y. and Hori, H. (2006) Functional categorization of the conserved basic amino acid residues in TrmH (tRNA (Gm18) methyltransferase) enzymes. *J. Biol. Chem.*, **281**, 34630–34639.
 46. Ochi, A., Makabe, K., Kuwajima, K. and Hori, H. (2010) Flexible recognition of the tRNA G18 methylation target site by TrmH methyltransferase through first binding and induced fit processes. *J. Biol. Chem.*, **285**, 9018–9029.
 47. Ny, T. and Björk, G.R. (1980) Growth rate-dependent regulation of transfer ribonucleic acid (5-methyluridine) methyltransferase in *Escherichia coli* B/r. *J. Bacteriol.*, **142**, 371–379.
 48. Persson, B.C., Gustafsson, C., Berg, D.E. and Björk, G.R. (1992) The gene for a tRNA modifying enzyme, m⁵U54-methyltransferase, is essential for viability in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **89**, 3995–3998.
 49. Urbonavicius, J., Jager, G. and Björk, G.R. (2007) Amino acid residues of the *Escherichia coli* tRNA(m⁵U54)methyltransferase (TrmA) critical for stability, covalent binding of tRNA and enzymatic activity. *Nucleic Acids Res.*, **35**, 3297–3305.
 50. Urbonavicius, J., Skouloubris, S., Myllykallio, H. and Grosjean, H. (2005) Identification of a novel gene encoding a flavin-dependent tRNA:m⁵U methyltransferase in bacteria: evolutionary implications. *Nucleic Acids Res.*, **33**, 3955–3964.
 51. Nishimasu, H., Ishitani, R., Yamashita, K., Iwashita, C., Hirata, A., Hori, H. and Nureki, O. (2009) Atomic structure of a folate/FAD-dependent tRNA T54 methyltransferase. *Proc. Natl Acad. Sci. USA*, **106**, 8180–8185.
 52. Yokoyama, S., Hirota, H., Kigawa, T., Yabuki, T., Shirouzu, M., Terada, T., Ito, Y., Mastuno, Y., Kuroda, Y., Nishimura, Y. et al. (2000) Structural genomics projects in Japan. *Nature Struct. Biol.*, **7**, 943–945.
 53. Hori, H., Yamazaki, N., Matsumoto, T., Watanabe, Y., Ueda, T., Nishikawa, K., Kumagai, I. and Watanabe, K. (1998) Substrate recognition of tRNA (Guanosine-2'-)-methyltransferase from *Thermus thermophilus* HB27. *J. Biol. Chem.*, **273**, 25721–25727.
 54. Keith, G. (1995) Mobilities of modified ribonucleotides on two-dimensional cellulose thin-layer chromatography. *Biochimie*, **77**, 142–144.
 55. Hoseki, J., Yano, T., Koyama, Y., Kuramitsu, S. and Kagamiyama, H. (1999) Directed evolution of thermostable kanamycin-resistance gene: a convenient selection marker for *Thermus thermophilus*. *J. Biochem.*, **126**, 951–956.
 56. Hoseki, J., Okamoto, A., Takada, N., Suenaga, A., Futatsugi, N., Konagaya, A., Taiji, M., Yano, T., Kuramitsu, S. and Kagamiyama, H. (2003) Increased rigidity of domain structures enhances the stability of a mutant enzyme created by directed evolution. *Biochemistry*, **42**, 14469–14475.
 57. Hashimoto, Y., Yano, T. and Kuramitsu, S. (2001) Disruption of *Thermus thermophilus* genes by homologous recombination using a thermostable kanamycin-resistant marker. *FEBS Lett.*, **506**, 231–234.
 58. Watanabe, K., Shinma, M., Oshima, T. and Nishimura, S. (1976) Heat-induced stability of tRNA from an extreme thermophile, *Thermus thermophilus*. *Biochem. Biophys. Res. Commun.*, **72**, 1137–1144.
 59. Awai, T., Kimura, S., Tomikawa, C., Ochi, A., Ihsanawati, Bessho, Y., Yokoyama, S., Ohno, S., Nishikawa, K., Yokogawa, T. et al. (2009) *Aquifex aeolicus* tRNA (N²,N²-Guanine)-dimethyltransferase (Trm1) catalyzes transfer of methyl groups not only to guanine 26 but also to guanine 27 in tRNA. *J. Biol. Chem.*, **284**, 20467–20478.
 60. Tomikawa, C., Yokogawa, T., Kanai, T. and Hori, H. (2010) N⁷-Methylguanine at position 46 (m⁷G46) in tRNA from *Thermus thermophilus* is required for cell viability through a tRNA modification network. *Nucleic Acids Res.*, **38**, 942–957.
 61. Yokogawa, T., Kitamura, Y., Nakamura, D., Ohno, S. and Nishikawa, K. (2010) Optimization of the hybridization-based method for purification of thermostable tRNAs in the presence of tetraalkylammonium salts. *Nucleic Acids Res.*, **38**, e89.
 62. Okamoto, H., Watanabe, K., Ikeuchi, Y., Suzuki, T., Endo, Y. and Hori, H. (2004) Substrate tRNA recognition mechanism of tRNA (m⁷G46) methyltransferase from *Aquifex aeolicus*. *J. Biol. Chem.*, **279**, 49151–49159.
 63. Droogmans, L., Roovers, M., Bujnicki, J.M., Tricot, C., Hartsch, T., Stalon, V. and Grosjean, H. (2003) Cloning and characterization of tRNA (m¹A58) methyltransferase (Trm1) from *Thermus thermophilus* HB27, a protein required for cell growth at extreme temperatures. *Nucleic Acids Res.*, **31**, 2148–2156.
 64. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L. and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.*, **2**, 2006–2008.
 65. Shigi, N., Sakaguchi, Y., Suzuki, T. and Watanabe, K. (2006) Identification of two tRNA thiolation genes required for cell growth at extremely high temperatures. *J. Biol. Chem.*, **281**, 14286–14306.
 66. De Bie, L.G., Roovers, M., Oudjama, Y., Wattiez, R., Tricot, C., Stalon, V., Droogmans, L. and Bujnicki, J.M. (2003) The *yggH* gene of *Escherichia coli* encodes a tRNA (m⁷G46) methyltransferase. *J. Bacteriol.*, **185**, 3238–3243.
 67. Shigi, N., Suzuki, T., Terada, T., Shirouzu, M., Yokoyama, S. and Watanabe, K. (2006) Temperature-dependent biosynthesis of 2-thioribothymidine of *Thermus thermophilus* tRNA. *J. Biol. Chem.*, **281**, 2104–2113.
 68. Shigi, N., Sakaguchi, Y., Asai, S., Suzuki, T. and Watanabe, K. (2008) Common thiolation mechanism in the biosynthesis of tRNA thiouridine and sulphur-containing cofactors. *EMBO J.*, **27**, 3267–3278.
 69. Yokoyama, S., Watanabe, K. and Miyazawa, T. (1987) Dynamic structures and functions of transfer ribonucleic acid from extreme thermophiles. *Adv. Biophys.*, **23**, 115–147.
 70. Mega, R., Manzoku, M., Shinkai, A., Nakagawa, N., Kuramitsu, S. and Masui, R. (2010) Very rapid induction of a cold shock protein by temperature downshift in *Thermus thermophilus*. *Biochem. Biophys. Res. Commun.*, **399**, 336–340.
 71. Bishop, A.C., Xu, J., Johnson, R.C., Schimmel, P. and de Crécy-Lagard, V. (2002) Identification of the tRNA-dihydrouridine synthase family. *J. Biol. Chem.*, **277**, 25090–25095.
 72. Dalluge, J.J., Hashizume, T., Sopchik, A.E., McCloskey, J.A. and Davis, D.R. (1996) Conformational flexibility in RNA: the role of dihydrouridine. *Nucleic Acids Res.*, **24**, 1073–1079.

73. Xing, F., Hiley, S.L., Hughes, T.R. and Phizicky, E.M. (2004) The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. *J. Biol. Chem.*, **279**, 17850–17860.
74. Rider, L.W., Ottosen, M.B., Gattis, S.G. and Palfey, B.A. (2009) Mechanism of dihydrouridine synthase 2 from yeast and the importance of modifications for efficient tRNA reduction. *J. Biol. Chem.*, **284**, 10324–10333.
75. Cavaille, J., Chetouani, F. and Bachellerie, J.P. (1999) The yeast *Saccharomyces cerevisiae* YDL112w ORF encodes the putative 2'-O-ribose methyltransferase catalyzing the formation of Gm18 in tRNAs. *RNA*, **5**, 66–81.