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N^7-Methylguanine at position 46 (m^7G46) in tRNA from *Thermus thermophilus* is required for cell viability at high temperatures through a tRNA modification network

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ABSTRACT

N^7-methylguanine at position 46 (m^7G46) in tRNA is produced by tRNA (m^7G46) methyltransferase (TrmB). To clarify the role of this modification, we made a *trmB* gene disruptant (*ΔtrmB*) of *Thermus thermophilus*, an extreme thermophilic eubacterium. The absence of TrmB activity in cell extract from the *ΔtrmB* strain and the lack of the m^7G46 modification in $tRNA^{Phe}$ were confirmed by enzyme assay, nucleoside analysis and RNA sequencing. When the *ΔtrmB* strain was cultured at high temperatures, several modified nucleotides in tRNA were hypo-modified in addition to the lack of the m^7G46 modification. Assays with tRNA modification enzymes revealed hypo-modifications of Gm18 and m^1G37, suggesting that the m^7G46 positively affects their formations. Although the lack of the m^7G46 modification and the hypo-modifications do not affect the Phe charging activity of $tRNA^{Phe}$, they cause a decrease in melting temperature of class I tRNA and degradation of $tRNA^{Phe}$ and $tRNA^{Ile}$. ^{35}S-Met incorporation into proteins revealed that protein synthesis in *ΔtrmB* cells is depressed above 70°C. At 80°C, the *ΔtrmB* strain exhibits a severe growth defect. Thus, the m^7G46 modification is required for cell viability at high temperatures via a tRNA modification network, in which the m^7G46 modification supports introduction of other modifications.

INTRODUCTION

To date, more than 100 modified nucleosides have been found in various RNA species (1,2). In particular, tRNA contains numerous modified nucleosides (3). All of these modified nucleosides are produced post-transcriptionally by specific tRNA modification enzymes or guide RNA systems (1). Among them, N^7-methylguanine at position 46 (m^7G46) in the variable region is one of the common modifications in tRNA and forms a tertiary base pair with the C13–G22 base pair (4,5).

The m^7G46 modification is generated by tRNA (m^7G46) methyltransferase [tRNA (guanine-N^7-)-methyltransferase, EC 2. 1. 1. 33; TrMet (m^7G46)] (1,6). It has been reported that the yeast enzyme is composed of two protein subunits (Trm8 and Trm82) and their genes have been identified (7). Bacterial genes have also been experimentally identified and named *trmB* (classical name, *yggH*) from *Escherichia coli* (8), *Bacillus subtilus* (9) and *Aquifex aeolicus* (10). There is a clear structural difference between eukaryotic and bacterial tRNA (m^7G46) methyltransferases: the eukaryotic enzyme is a heterodimer (7,11,12), while the bacterial enzyme is a monomer (8) or homodimer (9). Recently, crystal structures of the eukaryotic (13) and bacterial (9) enzymes have been reported. These structural studies strongly suggest that the substrate RNA recognition mechanisms differ considerably between the eukaryotic and bacterial enzymes, although both enzymes have a similar catalytic domain. In our previous study, we reported that the *A. aeolicus* TrmB recognizes the G46 base from the T-stem side in tRNA (10). Recently, we have reported that the C-terminal region, which is found

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in thermophilic bacterial enzymes, is required for protein stability at high temperatures and contributes to the selection of the precise guanine nucleotide (i.e. G46) to be modified (14). Furthermore, we investigated the RNA recognition mechanism of the yeast enzyme (Trm8–Trm82 complex) and found that yeast Trm8–Trm82 has more stringent recognition requirements for the tRNA molecule than *A. aeolicus* TrmB (15). Thus, protein structure–function relationship studies have been useful in the elucidation of the RNA recognition mechanism.

Functional studies of the m^7G46 modification have also recently been performed. Gene disruption mutants of yeast (7) or *E. coli* (8) have revealed that the tRNA (m^7G46) methyltransferase activity in yeast and *E. coli* is not essential for cell viability. However, it has been reported that a yeast double mutant strain lacking both the *trm*8 and *trm*4 genes showed rapid degradation of $tRNA^{Val}$ (16) [*trm*4 encodes yeast tRNA (m^5C34, 40, 48, 49) methyltransferase (17)]. Thus, the m^7G46 modification in yeast contributes to the stability of tRNA in conjunction with the other modified nucleotide(s) around the variable region in tRNA. Moreover, recently, we have reported that a gene involved in m^7G modification of tRNA is required for infection by the phytopathogenic fungus *Colletotrichum lagenarium* (18). In comparison with these eukaryotic enzymes, there is limited information about the bacterial enzyme. In the current study, we have focused on characterization of a *trmB* gene disruptant (*ΔtrmB*) strain of *Thermus thermophilus* HB8, an extreme thermophilic eubacterium. We report the importance of the m^7G46 modification for growth at high temperatures and propose a tRNA modification network, in which the m^7G46 modification has a positive effect on formation of other modifications in tRNA.

MATERIALS AND METHODS

Materials

[Methyl-^{14}C]-*S*-adenosyl-L-methionine (AdoMet) (1.95 GBq/mmol) was purchased from Perkin Elmer. Non-radioisotope labeled AdoMet was obtained from Sigma. DE52 is a product of Whatman. Q-Sepharose Fast Flow was bought from GE Healthcare. DNA oligomers were purchased from Invitrogen, and T7 RNA polymerase was from Toyobo. Other chemical reagents were of analytical grade.

Strain and media

The 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 $CaCl_2$ and 0.17 mM $MgCl_2$ after autoclaving. To make plates, gellan gum (Wako Pure Chemicals) was added to the medium (final concentration, 1.5%).

Selection of the target gene for disruption

We searched for the target gene in the *T. thermophilus* HB8 genome by BLAST-search using the amino acid sequence of the *E. coli* TrmB (classical name, YggH) (8). One target gene (TTHA1619), which was annotated as a gene encoding a methyltransferase of unknown function, was found to be a candidate for *T. thermophilus trmB*. The expected amino acid sequence of TTHA1619 shares high homology with *E. coli* and *A. aeolicus* TrmB proteins. The TTHA1619 gene was amplified by polymerase chain reaction (PCR) using the following primers: Tth TrmBN, 5′-GGG GCA TAT GCT GGT CGT GCC CGC CCG CCT CCA C-3′; Tth TrmBC, 5′-GGG GGA ATT CTT AGG TGT GGT CCT GGA CCA CCT C-3′. Underlined regions show restriction enzyme sites (Nde I and Eco RI). The amplified DNA was digested with Nde I and Eco RI, and ligated into the multi-cloning linker of pET30a *E. coli* expression vector. The expression of TTHA1619 protein in *E. coli* BL21 (DE3) Rosetta 2 strain was performed according to the manufacturer's manual. The protein was partially purified by heat-treatment and DE52 column chromatography according to the purification procedure for *A. aeolicus* TrmB (10). The enzymatic activity and modified nucleotide analysis were performed using yeast and *T. thermophilus* $tRNA^{Phe}$ transcripts as described in our previous report (14).

Construction of *ΔtrmB* (*Δ*TTHA1619) strain

The TTHA1619 gene was disrupted by replacement with the highly thermostable kanamycin nucleotidyltransferase (*HTK*) gene (19,20). The plasmid vector containing the TTHA1619 region disrupted by the *HTK* gene was purchased from RIKEN Biological Resource Center (Tsukuba, Japan) (21). *Thermus thermophilus* cells in late-log phase were transformed by the vector according to the report (22) and mutant colonies were selected on a plate containing 500 μg/ml kanamycin at 70°C. The genomic DNA from each colony was isolated, analyzed by PCR and Southern hybridization, and then the sequence of the replaced region was determined on ABI PRISM 310 DNA sequencers. Southern hybridization was performed at 55°C as reported previously (23). An alkaline phosphatase-labeled probe was prepared using the AlkPhos Direct Labeling system (GE Healthcare) and the hybridized bands were detected by monitoring the alkaline phosphatase activity consuming ECF substrate with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences).

Measurement of tRNA methyltransferase activities in S-100 and P-100 wash fractions

In this study, we used yeast and *T. thermophilus* $tRNA^{Phe}$ transcripts as standard substrates. The transcripts were prepared by using T7 RNA polymerase and purified by Q-Sepharose column chromatography and 10% polyacrylamide gel electrophoresis (PAGE) (7 M urea).

Cell extracts from the wild type and *Δ*TTHA1619 were prepared from late-log phase cells cultivated at 67°C for

in vitro methyl-transfer assay. Wet cells (0.3g) were suspended in 2 ml of buffer A [50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 50 mM KCl]. The cells were ground in a mortar with 0.15 g aluminum oxide and then the suspension was centrifuged at 8000*g* for 20 min. The supernatant fraction was further centrifuged at 100 000*g* for 2 h. The resultant supernatant was used as the S-100 fraction. Glycerol was added to the S-100 fraction to a final concentration of 50% v/v and stored at −30°C. The P-100 wash fraction was prepared from the precipitate of the centrifugation at 100 000*g*. The precipitate was homogenized with 200 µl of buffer A containing 1 M ammonium chloride and then centrifuged at 100 000*g* for 2 h. Subsequently, the supernatant was dialyzed against buffer A containing glycerol (final concentration 50% v/v) and used as the P-100 wash fraction.

Transfer RNA methyltransferase activities in the S-100 and P-100 wash fractions were analyzed as follows: 30 µg protein from the S-100 or P-100 wash fraction, 0.2 A_{260} unit yeast $tRNA^{Phe}$ transcript and 0.78 nmol [methyl-^{14}C]-AdoMet were incubated in 400 µl of buffer A at 60°C for 1 h. The RNA was extracted with phenol-chloroform and then recovered by ethanol precipitation. The RNA pellet was dissolved in 3 µl of 50 mM sodium acetate (pH 5.0), and digested with 2.5 units of nuclease P1 (Wako Pure Chemicals). The sample was separated using two dimensional thin layer chromatography (2D-TLC) as described previously (25). The ^{14}C-methylated nucleotides were monitored with a Fuji Photo Film BAS2000 imaging analyzer.

Nucleosides analysis by HPLC

Class I tRNA fractions were purified from the wild-type and *Δ*TTHA1619 cells in late-log phase cultured at 67°C. Briefly, total RNA fraction was prepared by phenol-chloroform extraction. Subsequently, the total RNA fraction was loaded on a Q-Sepharose column equilibrated with buffer B [20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 400 mM NaCl] and then a small RNA fraction (mainly tRNA and 5S rRNA) was eluted with buffer B containing 600 mM NaCl. The class I tRNA fraction was further purified by 10% PAGE (7 M urea). The class I tRNA fraction (0.2 A_{260} unit) was digested with 2 µg snake venom phosphodiesterase (Sigma), 20 µg RNaseA (Invitrogen), and 0.125 U bacterial alkaline phosphatase (Takara) in 20 µl of 50 mM Tris–HCl (pH 8.0) at 37°C overnight. Nucleosides were analyzed on an HPLC (Hitachi L-2000 system) equipped with a reverse phase C18 column (NUCLEOSIL 100 C18; 25 cm × 4.6 mm, 7 µm; GL Sciences, Inc). The solvent system consisted of buffer C [50 mM sodium phosphate (pH 5.1)] and buffer D (buffer C containing 70% methanol). The nucleosides (20 µl) were chromatographed using a flow rate of 1 ml/min with a multistep linear gradient as follows: 3% buffer D from 0 to 10 min, 3–35% D from 10 to 50 min, 35–98% B from 50 to 65 min, 98–100% B from 65 to 75 min and 100% buffer C from 75 to 85 min. Standard modified nucleosides [1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 2′-*O*-methyladenosine (Am), 2′-*O*-methylcytidine (Cm) and N^6-methyladenosine-5′-monophosphate (pm^6A)] were purchased from Sigma. The pm^6A nucleotide was dephosphorylated with bacterial alkaline phosphatase before use. 5-Methyl-2-thiouridine (m^5s^2U) was received as a kind gift from Dr Naoki Shigi (National Institute of Advanced Industrial Science and Technology, Japan). The elution points of 1-methylguanosine (m^1G), 2-methylguanosine (m^2G), 2′-*O*-methylguanosine (Gm), 7-methylguanosine (m^7G), 5-methyluridine (m^5U) and pseudouridine (Ψ) were determined by enzymatic formations using the tRNA modification enzymes, TrmD, Trm1, TrmH, TrmB, TrmA and TruB, respectively. The nucleoside contents quoted in Figure 5 were calculated as follows: the peak areas of all nucleosides were integrated and the ratio of each modified nucleoside was calculated. Subsequently, the ratio of each modified nucleoside in the *ΔtrmB* sample was divided by that in the wild-type sample.

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

3′-Biotinylated DNA oligomer (5′-TTC AGT CGC ATG CTC TAC CAA CT–biotin 3′) was used as a hybridization probe. The probe sequence is complementary from A36 to A14 of *T. thermophilus* $tRNA^{Phe}$. Purification of $tRNA^{Phe}$ by solid-phase DNA probe was performed as described in our previous report (25). The eluted $tRNA^{Phe}$ was further purified by 10% PAGE (7 M urea).

RNA sequencing

RNA sequences of the purified $tRNA^{Phe}$ (0.02 A_{260} unit) from the wild-type and *Δ*TTHA1619 strains were determined by Kuchino's post labeling method (26) with slight modifications as follows. Limited cleavage by formamide was performed at 90°C for 90 s, because the structure of *T. thermophilus* $tRNA^{Phe}$ is more stable as compared with tRNA species from mesophiles. For rapid detection of the modified nucleotides, we initially performed TLC using a single solvent system (isobutylic acid: conc. ammonia: water, 66:1:33, v/v/v). Later, we identified the modified nucleotides by 2D-TLC (24). 5′-^{32}P-labeled nucleotides were monitored with a Fuji Photo Film BAS2000 imaging analyzer. Standard nucleotides were marked by UV_{260nm} irradiation.

Growth phenotype analyses by plate culture

Thermus thermophilus wild-type and *ΔtrmB* strains were cultivated in rich medium at 67°C overnight. The diluted culture medium containing cells (A_{600} = 0.1) and sequential dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) (5 µl each) were spotted on to rich medium plates. These plates were incubated at 50°C for 50 h, at 60°C for 33 h, at 70°C for 13 h, at 75°C for 13 h and at 80°C for 15 h.

Translation activity analyses by ^{35}S-Met incorporation

The wild-type and *ΔtrmB* strains were cultured at 70°C. When the cell density ($A_{600\,nm}$) had reached 0.5, the culture medium (20 ml) was pre-incubated for 20 min at 80°C and then supplemented with 4.8 MBq of ^{35}S-Met. Culture medium (1 ml each) was sampled at various timepoints and the cells collected by centrifugation at

3500*g* for 2 min. The cells were washed with 500 µl of the medium and collected by centrifugation at 3500*g* for 2 min. The cells were resuspended in 20 µl of buffer A after which 20 µl of sodium dodecyl sulfate (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 21 500*g* for 5 min. The supernatants were analyzed by 15% SDS–PAGE. The gels were stained with Coomassie brilliant blue and ^{35}S-Met incorporation was monitored with a Fuji Photo Film BAS2000 imaging analyzer.

Melting profile analyses of class I tRNA and tRNA^{Phe}

The purified tRNA^{Phe} and class I tRNA fractions from the wild-type and *ΔtrmB* strains were prepared as described above. The tRNA^{Phe} transcript containing the m^{7}G46 modification was prepared by methylation of the tRNA^{Phe} transcript by *A. aeolicus* TrmB. Before the melting point measurement the tRNA fraction was annealed in buffer E [50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl] from 80 to 40°C for 60 min and then the melting curve was recorded on a spectrophotometer, UV-1650PC (Shimadzu). The temperature was increased from 20 to 95°C for 75 min. The melting profiles were obtained by averaging the two scans. The melting temperatures were calculated from first derivative plots.

Preparation of tRNA modification enzymes

Aquifex aeolicus TrmB was purified according to our previous report (10,14). *Thermus thermophilus* tRNA (Gm18) methyltransferase (TrmH) (23,27–30) and tRNA (m^{1}A58) methyltransferase (TrmI) (31) were kindly provided by Anna Ochi (Ehime University) and Masayuki Minoji (Ehime University), respectively. *Escherichia coli* tRNA m^{5}U54 methyltransferase (TrmA) (32–34) was a gift from Chikako Iwashita (Ehime University). *Aquifex aeolicus* tRNA (m^{1}G37) methyltransferase (TrmD) (35–37) was a gift from Mr Takashi Toyooka (Ehime University).

Aminoacylation of tRNA^{Phe}

The phenylalanine–tRNA synthetase (Phe-RS) fraction was prepared as follows. Briefly, the S-100 fraction (20 ml) of the wild-type strain was loaded onto a DE52 column (column volume, 10 ml) and Phe-RS was eluted by a KCl linear gradient (50–350 mM). The fractions containing Phe-RS were identified by Phe charging activity. The aminoacylation assay was performed using ^{14}C-Phe (18.4 GBq/mmol, Perkinelmer) as described in the reference (38). Phe charging activity was measured using 0.03A_{260} units of purified tRNA^{Phe} using a filter assay.

Northern hybridization

The small RNA fraction was purified by Q-Sepharose column chromatography as described above. The samples were separated by 10% PAGE (7 M urea), transferred to a membrane (Hybond-N+, GE Healthcare) by electro blotting, and fixed by $UV_{254\,nm}$ irradiation. Northern hybridization was performed with hybridization buffer (GE Healthcare) and 5′-^{32}P-labeled DNA probe at 52°C or 59°C [in the case of tRNA^{Val} (CAC)]. The DNA probe sequences are as follows: tRNA^{Phe} (UUC), 5′-TCA GTC GCA TGC TCT ACC AAC-3′; tRNA^{val} (CAC), 5′-AAC CGT GTG AGG CGA GCG CTC TT-3′; tRNA^{Arg} (CGG), 5′-CGG AGG CCG ACG CTC TAT C-3′; tRNA^{Tyr} (UAC), 5′-TAC AGA CCG TCC CCT TTG GC-3′; tRNA^{Ile} (AUC), 5′-ATC AGG CGT GCG CTC TAA CC-3′. The hybridized bands were monitored with a Fuji Photo Film BAS2000 imaging analyzer.

RESULTS

Construction of a potential *trmB* disruptant

In order to investigate the function of the m^{7}G46 modification in tRNA, we constructed a *trmB* disruptant strain of *T. thermophilus* HB8. We searched for the target gene in the *T. thermophilus* genome database by BLAST search using the amino acid sequence of *E. coli* TrmB. We identified the TTHA1619 gene as a candidate for *T. thermophilus trmB*. The expected amino acid sequence of TTHA1619 gene product shares high homology with both *E. coli* and *A. aeolicus* TrmB proteins (data not shown). The protein has a distinct basic amino acid rich region at its C-terminus, which is common to thermophilic TrmB proteins (14). To check the tRNA methyltransferase activity of the TTHA1619 gene product, we performed PCR cloning and inserted the amplified DNA into the pET30a *E. coli* expression vector. The recombinant protein was partially purified by heat treatment, and subsequently by DE52 column chromatography (data not shown). We confirmed tRNA methyltransferase activity by ^{14}C-methyl transfer assay using yeast and *T. thermophilus* tRNA^{Phe} transcripts, and ^{14}C-pm^{7}G formation activity by 2D-TLC (data not shown). On the basis of these experimental results, we selected the TTHA1619 gene as the target for gene disruption.

The plasmid vector for replacement of TTHA1619 by the *HTK* gene was purchased from RIKEN Biological Resource Center (21) and homologous recombination was performed according to the ref. (22). Colonies were isolated on a kanamycin plate and their genomic DNAs were prepared. The location of the *HTK* gene in the genome was analyzed by Southern hybridization. As a result, we were able to isolate a candidate *Δ*TTHA1619 strain (data not shown). We performed PCR cloning of the homologous recombination region and confirmed the DNA sequence (data not shown). These results confirmed that we had successfully selected a *Δ*TTHA1619 strain.

Absence of tRNA (m^{7}G) methyltransferase activity in cell extract from the *Δ*TTHA1619 strain

To confirm the absence of tRNA (m^{7}G) methyltransferase activity in the *Δ*TTHA1619 strain, we prepared S-100 and P-100 wash fractions from the wild-type and *Δ*TTHA1619 strains. The S-100 fraction from the wild-type or *Δ*TTHA1619 strain, yeast tRNA^{Phe} transcript, and ^{14}C-AdoMet were incubated at 60°C for 1 h. The RNA

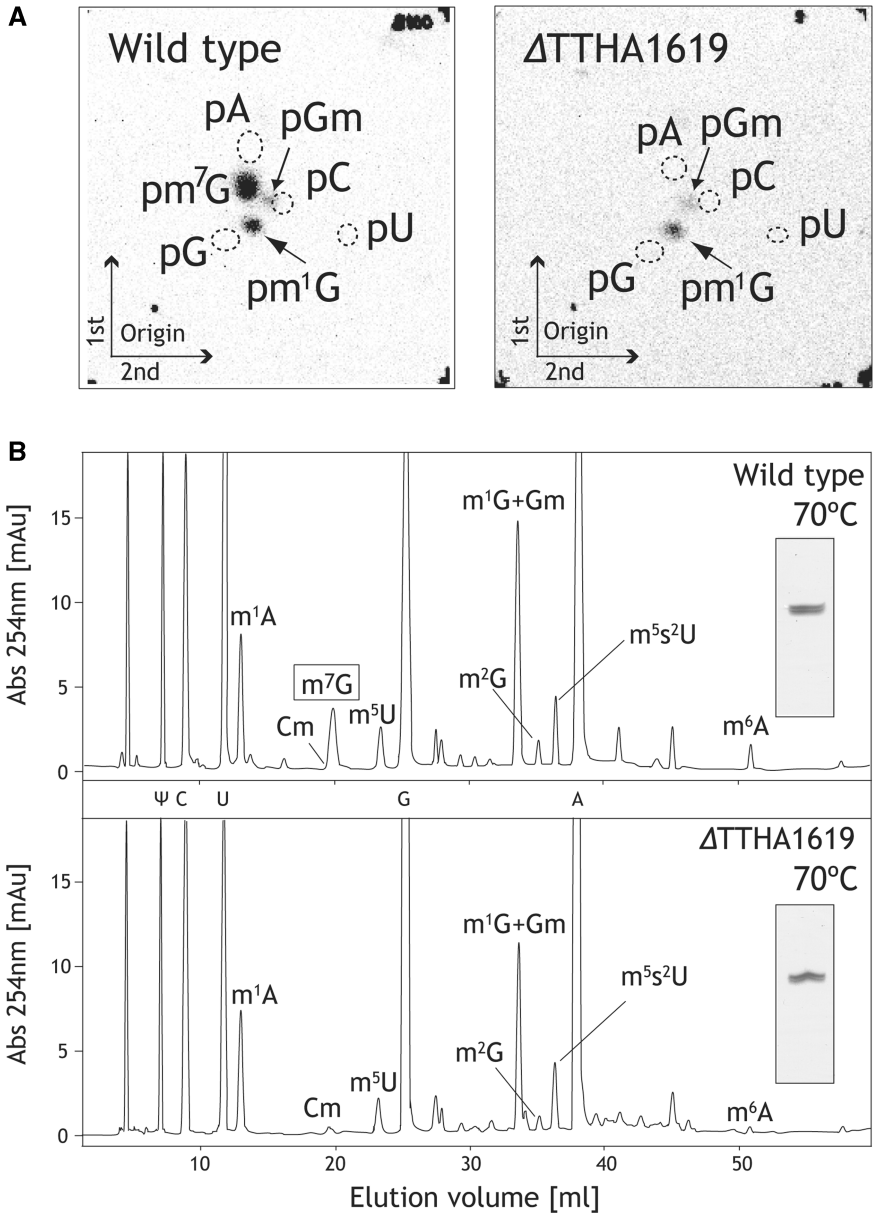

Figure 1. Absence of m^7G formation activity and lack of m^7G nucleoside in extract and tRNA from the *Δ*TTHA1619 strain. (**A**) yeast $tRNA^{Phe}$ transcript, [methyl-^{14}C]-AdoMet and S-100 fraction of the wild-type (left) or *Δ*TTHA1619 (right) strain were incubated at 60°C for 1 h, and ^{14}C-methylated nucleotides were analyzed by 2D-TLC. Positions of standard markers (pA, pG, pC and pU) are enclosed by dotted circles. (**B**) nucleoside analyses of the class I tRNA fractions from the wild-type (upper) and *Δ*TTHA1619 (lower) strains. 0.03 A_{260} units of the purified class I tRNA fractions were analyzed by 10% PAGE (7 M urea) and the gel was stained with toluidine blue (insets).

was recovered by ethanol precipitation and digested with nuclease P1. The ^{14}C-labeled mononucleotides were analyzed by 2D-TLC (Figure 1A). Left panel of Figure 1A shows the result from the wild-type strain. A ^{14}C-pm^7G spot was clearly detected in addition to spots representing ^{14}C-pm^1G and ^{14}C-pGm (^{14}C-pm^1G and ^{14}C-pGm were probably derived from TrmD and TrmH activities, respectively). It should be mentioned that TrmI was mainly present in the P-100 wash fraction (data not shown) and its activity was barely detected in the S-100 fraction (Figure 1A). Furthermore, in the case of *T. thermophilus*, the m^5U54 modification is generated by the 5,10-methylenetetrahydrofolate-dependent tRNA methyltransferase, TrmFO (39,40). Thus, ^{14}C-pm^5U was not detected in this experiment, whilst pm^7G, pm^1G and pGm were identified as ^{14}C-labeled mononucleotides in the wild-type sample. In contrast, the ^{14}C-pm^7G spot was completely absent from the *Δ*TTHA1619 sample (Figure 1, right panel), demonstrating that the tRNA (m^7G) methyltransferase activity was not present in the S-100 fraction from the *Δ*TTHA1619 strain. Furthermore, analysis of the AdoMet-dependent tRNA methyltransferase activities in the P-100 wash fractions confirmed that the tRNA (m^7G) methyltransferase activity was not observed in the fraction from the *Δ*TTHA1619 strain (data not shown). Taking these

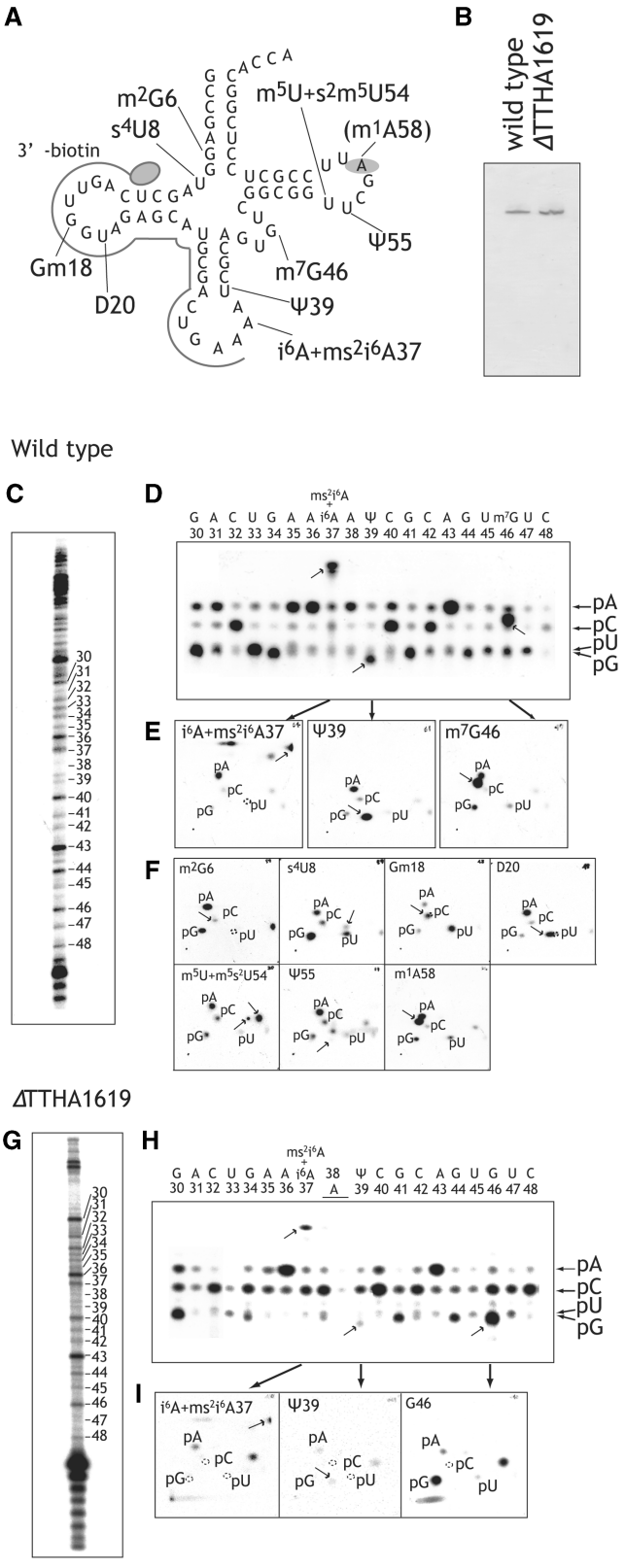

Figure 2. Sequence analyses of purified $tRNA^{Phe}$ from the wild-type and *Δ*TTHA1619 strains by Kuchino's post labeling method. (**A**) Nucleotide sequence of *T. thermophilus* $tRNA^{Phe}$ is depicted as a cloverleaf structure. The 3′-biotin DNA probe is illustrated. The m^1A58 modification was identified in this work. (**B**) 0.01 A_{260} units of purified $tRNA^{Phe}$ from the wild-type (left) and *Δ*TTHA1619 (right) strains were analyzed by 10% PAGE (7 M urea). The gel was stained by toluidine blue. The purified $tRNA^{Phe}$ from the wild-type (**C**) and *Δ*TTHA1619 (**G**) strains was partially cleaved by formamide. Then the 5′-end of each fragment was labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. The RNA fragments were separated by 15% PAGE (7M urea). Numbers correspond to the nucleotide positions in $tRNA^{Phe}$. The $tRNA^{Phe}$ fragments of the wild-type (**D**) and *Δ*TTHA1619 (**H**) strains were cut off from the gels in panels C and G, respectively. The fragments were digested with nuclease P1 and their 5′-nt were analyzed by TLC. In panels D and H, nucleotides at positions from 30 to 48 are shown. Positions of standard markers (pA, pG, pC and pU) are indicated by arrows at the right side of the thin layer plates. (**E**, **I**) Modified nucleotides of panels D and H were analyzed by 2D-TLC. The arrows indicate spots of modified nucleotides. (**F**) TLC patterns of the all modified nucleotides identified in $tRNA^{Phe}$ from the wild-type strain are shown.

experimental results together, we concluded that the tRNA (m^7G) methyltransferase activity was absent from the cell extract of the *Δ*TTHA1619 strain.

Absence of the m^7G modification in the Class I tRNA fraction from the *Δ*TTHA1619 strain

Next, we performed nucleoside analysis of the class I tRNA fraction, because the m^7G46 modification is not found in class II tRNA species. The class I tRNA fractions from the wild-type and *Δ*TTHA1619 strains were purified as shown in Figure 1B insets. Figure 1B upper panel shows the result of the nucleoside analysis from the wild-type strain. All labeled modified nucleosides in Figure 1B were experimentally identified by comparison with standard markers or enzymatic formation as described in experimental procedures (data not shown). The m^7G nucleoside eluted at 20.0 ml. In contrast, the peak representing m^7G was missing from the *Δ*TTHA1619 sample (Figure 1B, lower). Thus, we confirmed the absence of the m^7G46 modification in class I tRNA from the *Δ*TTHA1619 strain.

As well as the alteration described above we also noticed changes in other modifications of class I tRNA from the *Δ*TTHA1619 strain. As shown in Figure 1B, the content of the m^1G + Gm and m^6A seemed to be decreased. Because m^1G and Gm eluted at the same point (32.4 min) on our HPLC system, they could not be distinguished. The m^6A modification is generally produced in two ways. One is derivation from the m^1A58 modification produced by TrmI, through non-enzymatic conversion of m^1A to m^6A. Another is the m^6A37 modification, for which the responsible enzyme has not been identified. Moreover, at least, three modifications (Gm18, m^5s^2U54 and m^1A58) in *T. thermophilus* tRNA are often not complete and the contents of these modifications in tRNA change according to the culture conditions (especially culture temperature) (41–44). Therefore, we carefully analyzed modification rates of these changes in *ΔtrmB* strain. The detailed analyses of these modifications are described in a later section.

Absence of the m^7G46 modification in purified $tRNA^{Phe}$

The sequence of $tRNA^{Phe}$ has been reported (Figure 2A) (41). In *T. thermophilus* $tRNA^{Phe}$, the m^7G modification exists only at position 46. To confirm the absence of this

modification in $tRNA^{Phe}$ from the ΔTTHA1619 strain, we purified $tRNA^{Phe}$ by the solid phase DNA probe method, which was recently reported (25). The DNA probe sequence was designed to be complementary to the RNA sequence from the D-loop to the anti-codon loop (Figure 2A), because the sequence in this region of $tRNA^{Phe}$ is quite different from those of the other *T. thermophilus* tRNA species. To elute the RNA efficiently, tetramethylammonium chloride was selected as the salt in the hybridization buffer. This approach was successful in purifying $tRNA^{Phe}$ from tRNA mixtures of the wild-type and ΔTTHA1619 strains (Figure 2B). We further purified the $tRNA^{Phe}$ by 10% PAGE (7 M urea).

To determine precise positions of the modified nucleosides, we performed RNA sequencing. In these experiments, we selected Kuchino's post label method (26) to visualize the modifications. Briefly, the purified $tRNA^{Phe}$ was partially cleaved by formamide, and then the 5′-end of each fragment was labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. The RNA fragments were separated by 15% PAGE (7 M urea) (Figure 2C and G), cut off, extracted and recovered by ethanol precipitation. The recovered RNA was completely digested with nuclease P1 and ^{32}P-labeled nucleotides were analyzed by TLC. Because the RNA sequence of *T. thermophilus* $tRNA^{Phe}$ has been reported (41), we initially performed one-dimensional TLC to search for modified nucleotides rapidly (Figure 2D and H), and then modified nucleotides were analyzed by the standard 2D-TLC method (Figure 2E, F and I). Figure 2C–F shows the results obtained from the $tRNA^{Phe}$ of the wild-type strain. In Figure 2C and D, nucleotide positions from 30 to 48 can be seen. In this region, the presence of three modified bases [i^6A37 (partially modified to ms^2i^6A37), Ψ39, and m^7G46] has been reported (Figure 2A). In fact, these modifications could be detected in our purified $tRNA^{Phe}$ from the wild-type strain (Figure 2D and E). In the same way, we analyzed the other positions of the purified $tRNA^{Phe}$ from the wild-type strain and we could detect from m^2G6 to A73 in the $tRNA^{Phe}$ (data not shown). As a result, seven additional modifications [m^2G6, s^4U8, Gm18, D20, m^5U54 (partially modified to m^5s^2U54), Ψ55 and m^1A58] could be detected (Figure 2F), consistent with nucleosides analysis of the purified $tRNA^{Phe}$ by HPLC (data not shown). It should be mentioned that the m^1A58 modification in *T. thermophilus* $tRNA^{Phe}$ has not been previously reported (41). As described above, the content of the m^1A58 modification in tRNA changes according to the culture conditions (41–44). In the current study, we purified $tRNA^{Phe}$ from cells cultured at 70°C under 1 l per 1 min air supply into 1 L rich medium. The difference in the m^1A58 modification between previous studies and those described here may be caused by differences in the culture conditions and/or purification procedures.

Next, we analyzed the $tRNA^{Phe}$ from the ΔTTHA1619 strain (Figure 2G–I). As expected, position 46 was found to be unmodified G46 (Figure 2H and I). These experimental results clearly showed that the TTHA1619 gene is the *T. thermophilus trmB*. Hereafter, we describe ΔTTHA1619 as *ΔtrmB*.

Growth defect of the *ΔtrmB* strain at high temperatures

We examined the growth phenotype of the *ΔtrmB* strain at various temperatures. Figure 3A shows the results of plate cultures at 50, 60, 70, 75 and 80°C. The wild-type strain can live at a wide range of the temperature (50–80°C) although growth at 50°C is very slow (Figure 3A). Below 60°C, the wild-type and *ΔtrmB* strains exhibited similar growth. However, above 70°C, a growth defect in the *ΔtrmB* strain was observed. The *ΔtrmB* strain showed a long lag phase at 70°C as compared with the wild type (Figure 3A) although the growth curve of the *ΔtrmB* strain in liquid culture was almost the same as that of the wild type (Figure 3B). When the *ΔtrmB* strain was cultured at 75 or 80°C, the growth defect was more clearly observed. At 80°C in particular, growth on a plate was not observed within 15 h (Figure 3A) and the growth in liquid culture showed a severe growth defect (Figure 3C). In fact, bacteriolysis is often observed during culture at 80°C: Figure 3C shows one example in which cell number began to decrease around the 12 h mark. These experimental results suggested that the m^7G46 modification in tRNA is required for viability of *T. thermophilus* at high temperatures.

We tested several culture conditions for preparation of tRNA and proteins at high temperatures. As a result, we found that the *ΔtrmB* strain could survive at 80°C in liquid culture when it was cultured at 70°C until the middle log phase and then the culture temperature could be shifted from 70 to 80°C (Figure 3D). The existence of TrmB seems to be more important in lag and early log phases than after middle log phase. Hereafter in this article, we describe these culture conditions as 70–80°C.

^{35}S-Met incorporation into proteins in the *ΔtrmB* strain decreased not only at 80°C but also at 70°C

We compared *in vivo* protein synthesis activities of the wild-type and *ΔtrmB* strains by monitoring ^{35}S-Met incorporation. When the cell density had reached $0.5A_{600\,nm}$, ^{35}S-Met was directly added into the culture medium. Figure 4A shows the results at 70°C. Unexpectedly, the speed of ^{35}S-Met incorporation into proteins in the *ΔtrmB* cells was clearly slowed as compared with that in the wild-type cells. Thus, although the apparent growth curves of both strains in liquid culture are similar at 70°C, protein synthesis activity of the *ΔtrmB* strain is lower than that of the wild-type strain even at 70°C. At 70°C, protein synthesis is not rate limiting unlike other processes such as DNA replication. In contrast, ^{35}S-Met incorporation of the *ΔtrmB* cells cultured at 70–80°C is clearly inferior to that of the wild-type cells (Figure 4B), consistent with the growth phenotypes. These experimental results demonstrate that the lack of m^7G46 modification in tRNA causes depression of protein synthesis at high temperatures.

Other modifications are decreased in the class I tRNA fraction from the *ΔtrmB* cells cultured at 70–80°C

We have observed decreases in some modifications in tRNA from the *ΔtrmB* strain at 70°C as described

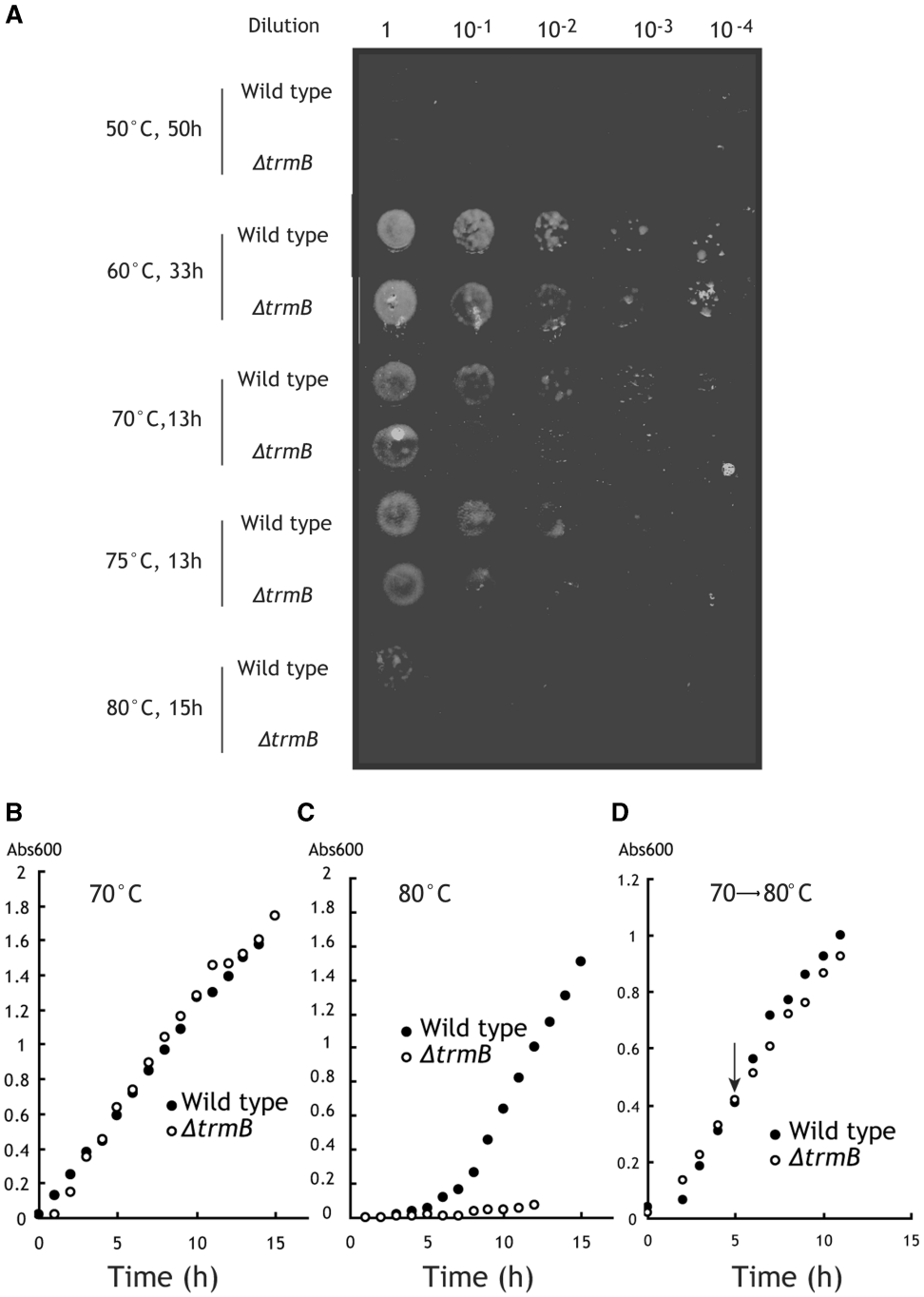

Figure 3. Growth phenotypes of the wild-type and *ΔtrmB* strains. (**A**) The wild-type and *ΔtrmB* strains were serially diluted, spotted onto plates containing rich medium, and incubated at the temperatures indicated. Incubation time is indicated next to temperature. The growth curves of the wild-type and *ΔtrmB* strains in liquid cultures at 70°C (**B**), 80°C (**C**) and 70–80°C (**D**). The arrow in panel D indicates the shift point of temperature from 70°C to 80°C.

above. To clarify whether the lack of the m^7G46 modification was having effects on other modifications at high temperatures, we prepared class I tRNA fractions from the wild-type and *ΔtrmB* cells cultured at 70–80°C. Figure 5 shows results of the nucleosides analyses. As expected, the amounts of various modified nucleosides were decreased in the *ΔtrmB* sample in addition to the disappearance of m^7G (Figure 5, lower). Thus, we confirmed that the content of Ψ, m^2G, m^5U, m^6A and m^1G + Gm in the class I tRNA from the *ΔtrmB* cells cultured at 70–80°C decreased compared with those from the wild-type cells.

Assays with tRNA modification enzymes demonstrate the hypo-modifications in Class I tRNA from the *ΔtrmB* strain

Hypo-modifications in class I tRNA from the *ΔtrmB* strain suggested that the lack of the m^7G46 modification

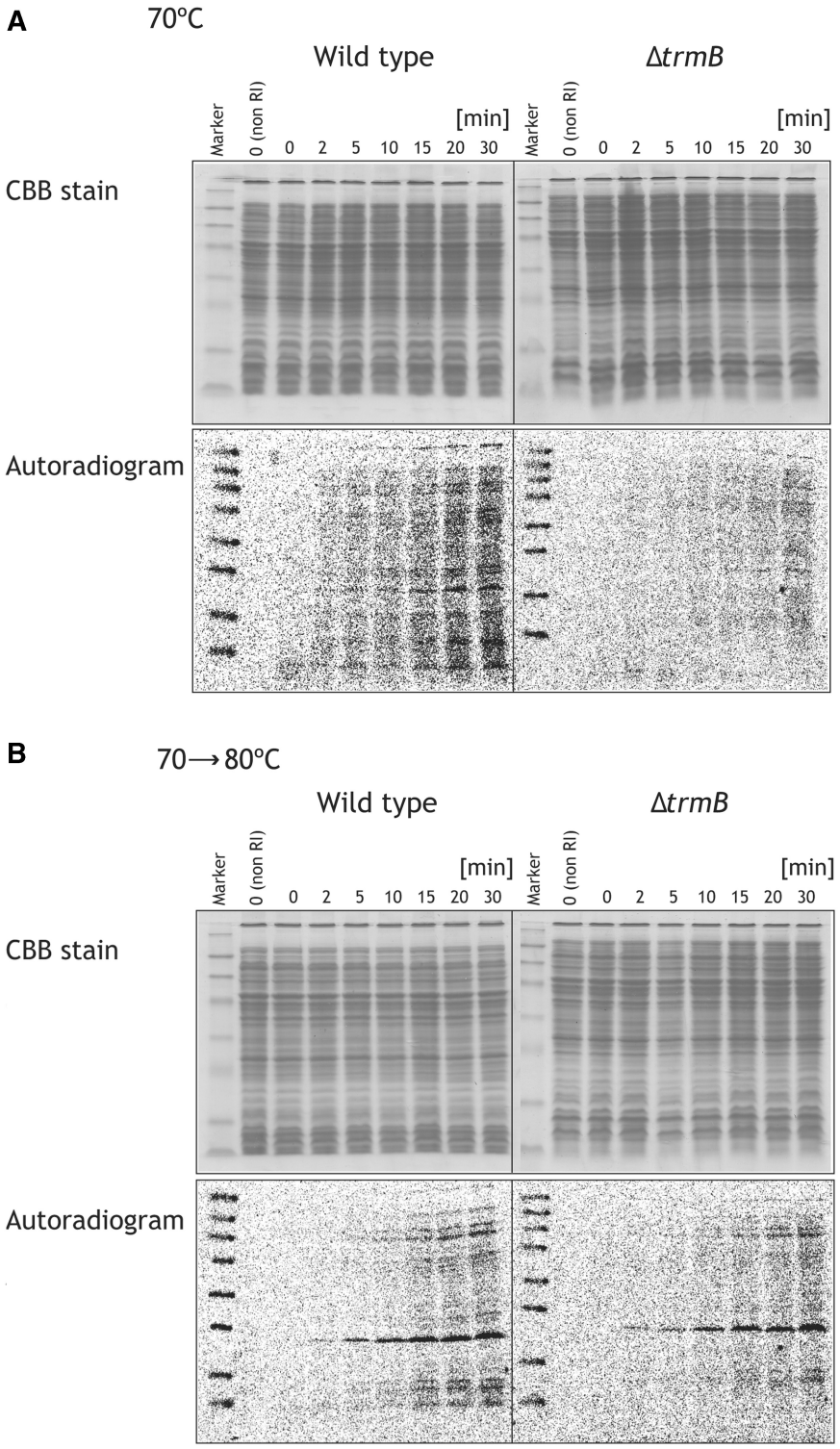

Figure 4. Protein synthesis activities in the wild-type and *ΔtrmB* strain. The protein synthesis activities of the wild-type (left) and *ΔtrmB* (right) strains were compared by ^{35}S-Met incorporation at 70°C (**A**) and 70–80°C (**B**). ^{35}S-Met was added at the zero points and samples were taken out at 2, 5, 10, 15, 20 and 30 min. Total proteins were analyzed by 15% SDS–PAGE. The gels were stained with Coomassie brilliant blue (upper). ^{35}S-Met incorporation was monitored with a Fuji-photo film imaging analyzer (lower). Non RI means the sample before addition of ^{35}S-Met.

affects the activities of several tRNA modification enzymes. To confirm this idea, we performed assays using tRNA modification enzymes. The Gm18, m^1G37, m^5U54 and m^1A58 modifications can be produced by TrmH, TrmD, TrmA and TrmI, respectively (Figure 6A). If these modifications decreased in tRNA from the *ΔtrmB* strain, the tRNA would be a better substrate for these enzymes as compared to the tRNA

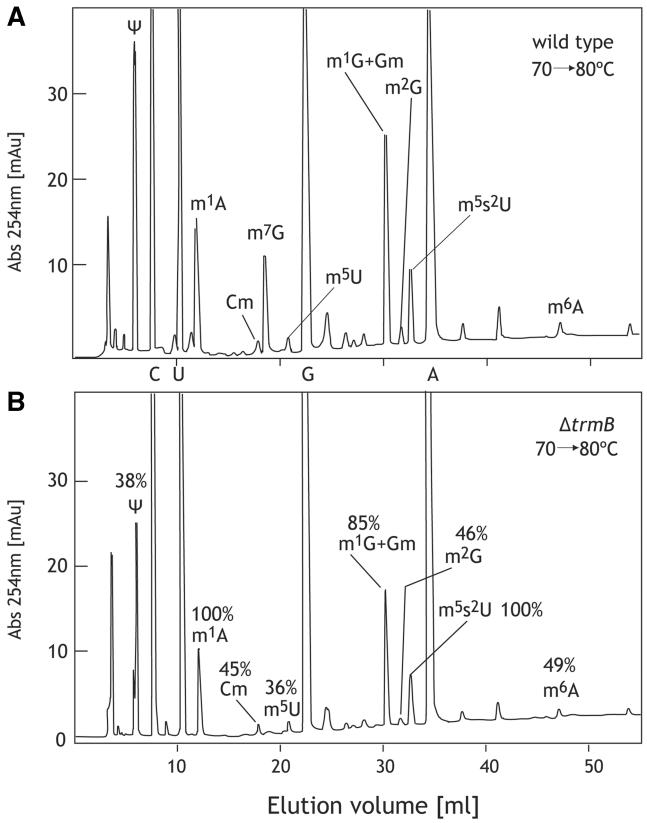

Figure 5. The contents of modified nucleosides in class I tRNA from the *ΔtrmB* cells cultured at 70–80°C decrease. The modified nucleosides in class I tRNA from the wild-type (**A**) and the *ΔtrmB* (**B**) cells cultured at 70–80°C were compared. The content of each modified nucleosides was calculated as described in the 'Materials and methods' section, and is depicted in the figure.

from the wild-type strain. We prepared five tRNA modification enzymes (*T. thermophilus* TrmH and TrmI, *A. aeolicus* TrmB and TrmD, and *E. coli* TrmA) (Figure 6B). In *T. thermophilus*, the m^5U54 modification is generated by 5,10-methylenetetrahydrofolate-dependent tRNA methyltransferase TrmFO (39), however 5,10-methylenetetrahydrofolate is unstable and the radioisotope labeled compound is not commercially available. Therefore, we prepared the *E. coli* TrmA protein instead of TrmFO.

Total RNAs from the wild-type and *ΔtrmB* strains cultured at 70°C were prepared. To clarify the role of the m^7G46 modification, we modified total RNA from the *ΔtrmB* strain with *A. aeolicus* TrmB (we prepared the total RNA from the *ΔtrmB* strain with m^7G46 modification by *in vitro* enzymatic formation). Methyl group acceptance activities of these RNAs were examined using the tRNA modification enzymes. In the case of *E. coli* TrmA, the assay was performed at 37°C. Figure 6C shows the ^{14}C-methyl group incorporation at the 60 min period. The total RNA from the *ΔtrmB* strain is well methylated by *A. aeolicus* TrmB, while that from the wild-type strain is scarcely methylated, consistent with the lack of the m^7G46 modification in the *ΔtrmB* strain. *E. coli* TrmA did not methylate total RNA from the wild-type strain as well as that from the *ΔtrmB* strain, demonstrating that U54 is nearly completely modified to m^5U54 or m^5s^2U54 in both the wild-type and *ΔtrmB* strains. *A. aeolicus* TrmD methylated the RNA from the *ΔtrmB* strain. Thus, a fraction of the G37 in the *ΔtrmB* RNA was not completely modified in the cells. Furthermore, when G46 was modified to m^7G46 by *A. aeolicus* TrmB, the velocity of the m^1G37 modification by TrmD was slightly increased. Although the source of the TrmD was *A. aeolicus*, the presence of the G46 modification increased m^1G37 formation by TrmD. This tendency was more clearly observed with the Gm18 modification. In the case of the Gm18 modification, time-dependent experiments were performed to compare the velocities (Figure 6D). G18 in the RNA from the wild-type strain is near fully modified to Gm18, as confirmed by the fact that the RNA was scarcely modified by TrmH. In contrast, the RNA from the *ΔtrmB* strain was efficiently methylated by TrmH, suggesting that G18 in the *ΔtrmB* strain is hypo-modified. Furthermore, *in vitro* introduction of m^7G46 clearly accelerated Gm18 formation. Moreover, this tendency was also observed with the m^1A58 modification by TrmI, although A58 is considerably modified to m^1A58 in both the wild-type and *ΔtrmB* cells (Figure 6C).

These experimental results suggest that there is a tRNA modification network, in which the m^7G46 modification has a positive effect on other modifications. In *T. thermophilus*, the m^7G46 modification by TrmB enhances the formation velocities of Gm18 by TrmH, m^1G37 by TrmD and m^1A58 by TrmI. The lack of the m^7G46 modification causes hypo-modifications of Gm18 and m^1G37 (m^1A58 is slightly hypo-modified). In the nucleoside analysis (Figure 5B), the contents of Ψ, m^2G, m^5U, m^6A and m^1G + Gm decreased. The Ψ modifications are introduced into various positions by multiple enzymes. The enzymes responsible for the m^2G6 and m^6A37 modifications have not been identified. Therefore, the effect of the m^7G46 modification on these enzymes could not be experimentally verified. However, our experimental results demonstrated that the presence of the m^7G46 modification induces nearly full modification of several modified nucleotides such as Gm18 and m^1G37.

Aminoacylation of purified $tRNA^{Phe}$

Protein synthesis activity of the *ΔtrmB* strain is low compared with that of wild-type strain at 70°C as well as at 70–80°C. The proportion of some modified nucleotides in Class I tRNA from the *ΔtrmB* strain are low compared with those from the wild-type strain. Do these hypo-modifications affect amino acid charging activity of tRNA? We partially purified the Phe-RS fraction and measured the Phe charging activity of $tRNA^{Phe}$. In these experiments, we purified $tRNA^{Phe}$ from wild-type and *ΔtrmB* cells cultured at 70 and 70–80°C. Figure 7 shows the aminoacylations at 70°C (left) and 80°C (right) of the purified $tRNA^{Phe}$ from wild-type and *ΔtrmB* cells cultured at 70°C. As shown in Figure 7, there is no difference between Phe charging velocities of the purified $tRNA^{Phe}$ from wild-type and

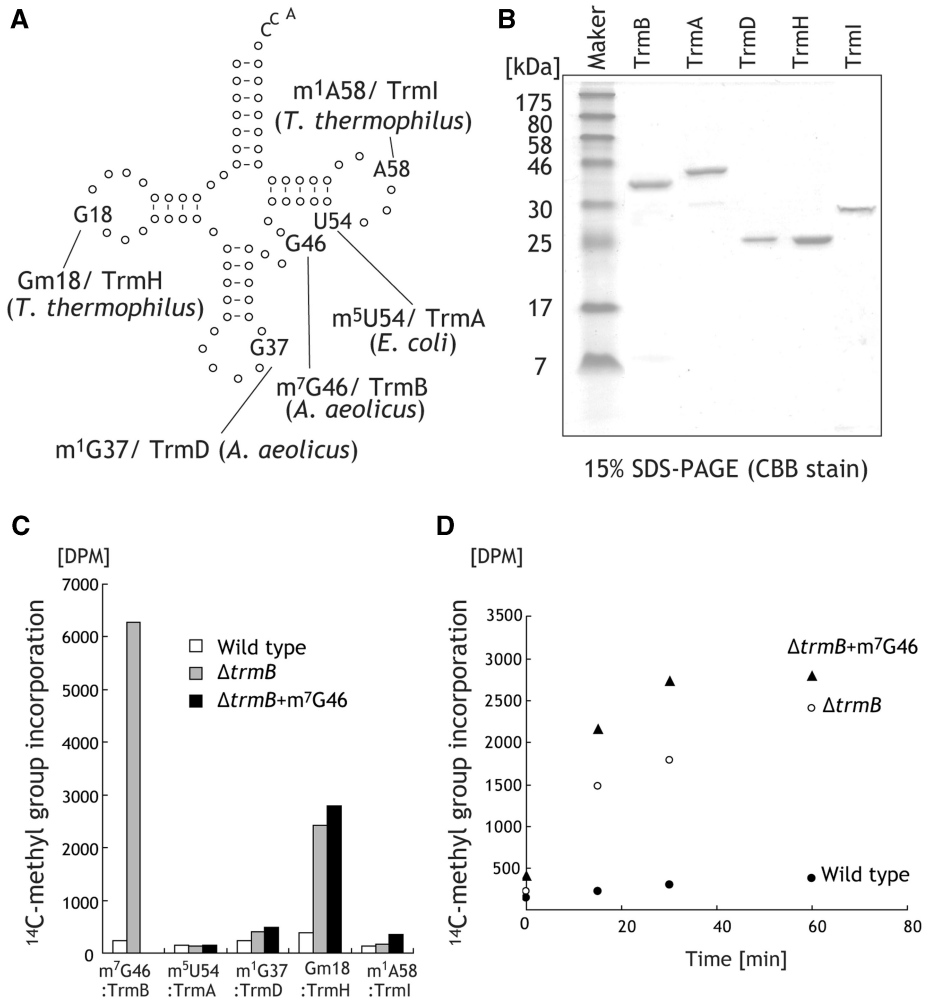

Figure 6. Methyl-transfer activities of tRNA methyltransferases for the RNAs from the wild-type and *ΔtrmB* strains. (**A**) Modified nucleotides are indicated in a tRNA cloverleaf structure together with the responsible modification enzymes (TrmB, TrmA, TrmD, TrmH and TrmI). Sources of the enzymes are indicated in parenthesis. (**B**) Purified tRNA methyltransferases (TrmB, TrmA, TrmD, TrmH and TrmI) were analyzed by 15% SDS–PAGE and the gel was stained with Coomassie brilliant blue. (**C**) ^{14}C-methyl group acceptance activity of RNAs from the wild-type (white) and *ΔtrmB* (grey) strain. The *ΔtrmB* + m^{7}G46 (black) annotation on the graph means the methyl acceptance activity of the RNA from the *ΔtrmB* strain methylated with TrmB. The methyl group acceptance activities at a 60 min period are shown. With the exception of the Gm18 modification by TrmH the graphs represent the apparent initial velocities. The methylation velocities except that for the Gm18 modification were relatively slow because the modified tRNA, which is abundant in the RNA, inhibits the modifications. (**D**) Methyl group incorporation catalyzed by TrmH was monitored in a time-dependent manner.

the *ΔtrmB* strain at 70 and 80°C. Furthermore, we examined Phe charging activity of the tRNA^{Phe} from the wild-type and the *ΔtrmB* cells cultured at 70–80°C and confirmed that Phe charging velocities were the same at 70 and 80°C (data not shown). Thus, the lack of the m^{7}G46 and the presence of hypo-modifications do not affect the Phe charging activity of tRNA^{Phe}.

Melting temperatures of the class I tRNA fractions from the *ΔtrmB* strain decrease

The lack of m^{7}G46 and hypo-modifications of other modified nucleotides does not affect amino acid charging activity (at least in the case of Phe charging activity); however, protein synthesis activity of the *ΔtrmB* strain is clearly inferior to that of the wild type. To address this problem, we measured melting temperatures of class I tRNA fractions from the wild-type and *ΔtrmB* strains (Table 1). To clarify the contribution of the m^{7}G46 modification to melting temperature, we prepared yeast tRNA^{Phe} transcript containing m^{7}G46 by *in vitro* methyl-transfer reaction with *A. aeolicus* TrmB. As shown in Table 1, the presence of the m^{7}G46 modification increased the melting temperature by only 0.1°C. However, class I tRNA fractions showed a clear difference in melting temperatures. The melting temperature of class I tRNA from the *ΔtrmB* strain was 76.2°C while that from the wild-type strain was 79.7°C. Thus, the lack of m^{7}G46 and hypo-modifications cause a 3.5°C decrease in the melting temperature. At 80°C, some tRNA species from the *ΔtrmB* strain may be structurally loosened. Furthermore, we measured melting temperatures of the class I tRNA

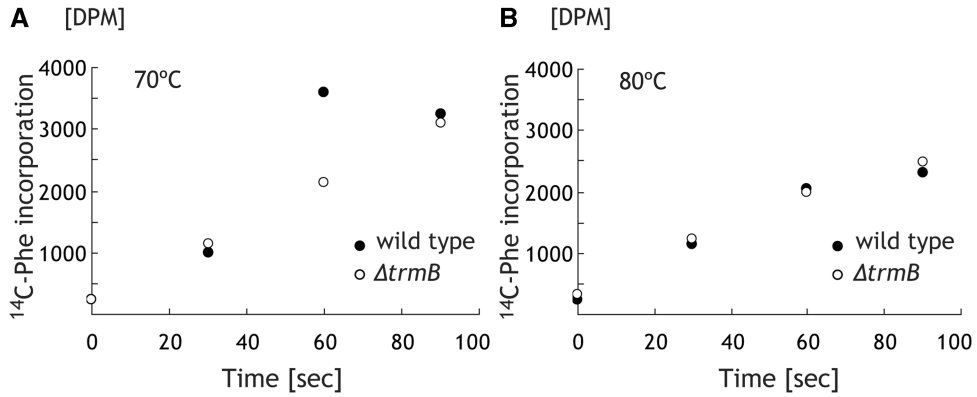

Figure 7. Phe charging activities of purified $tRNA^{Phe}$. Phe charging activities at 70°C (**A**) and 80°C (**B**) were measured by ^{14}C-Phe incorporation into the purified $tRNA^{Phe}$ from the wild-type (filled circles) and *ΔtrmB* (open circles) strains. These graphs show one set of data from two independent experiments.

Table 1. Melting temperatures of tRNA

tRNA	Melting temperature (°C)
yeast $tRNA^{Phe}$ transcript	71.6
yeast $tRNA^{Phe}$ transcript containing m^7G46	71.7
Class I tRNA	
70°C Wild-type	79.7
70°C *ΔtrmB*	76.2
70–80°C Wild-type	79.7
70–80°C *ΔtrmB*	78.5

fractions from the wild-type and *ΔtrmB* cells cultured at 70–80°C. Unexpectedly, the melting temperature of the class I tRNA fraction of the *ΔtrmB* strain increased to 78.5°C (Table 1). In spite of enhancement of the hypo-modifications, the melting temperature increased. This experimental result prompted us to investigate the tRNA population.

Degradation of some kinds of tRNA species in the *ΔtrmB* cells cultured at 70–80°C

We performed northern hybridization to investigate the tRNA population (Figure 8). As shown in Figure 8A, small RNA (mainly tRNA and 5S rRNA) fractions were prepared from the wild-type and the *ΔtrmB* cells cultured at 70–80°C. In the experiments, zero time refers to the start point of the temperature shift to 80°C. We prepared four class I tRNA probes and one class II tRNA probe ($tRNA^{Tyr}$). These class I tRNA species contain the m^7G46 modification in the wild-type strain. As shown in Figure 8B, the class I tRNA species in the wild-type cells did not decrease within 6 h: $tRNA^{Phe}$ and $tRNA^{Arg}$ slightly increased and $tRNA^{Tyr}$ slightly decreased. In contrast, $tRNA^{Phe}$ and $tRNA^{Ile}$ in the *ΔtrmB* cells clearly decreased (Figure 8B). Thus, populations of tRNA species in the *ΔtrmB* cells are changed through degradation. These experimental results explain the increase in the melting temperature of the class I tRNA from the *ΔtrmB* cells cultured at 70–80°C.

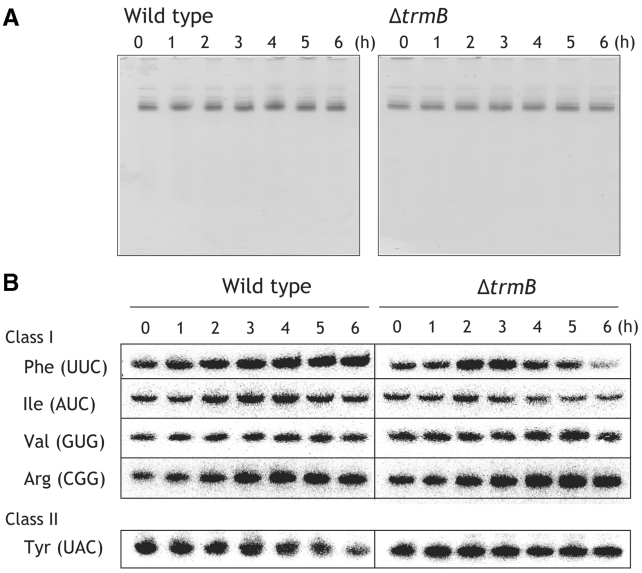

Figure 8. Degradation of $tRNA^{Phe}$ (UUC) and $tRNA^{Ile}$ (AUC) in the *ΔtrmB* cells cultured at 70–80°C. (**A**) Small RNA fractions of the wild-type (left) and *ΔtrmB* (right) strains were time-dependently prepared from the cells cultured at 70–80°C. The zero periods mean the shift points of the culture temperature from 70°C to 80°C. 0.02 A_{260} units of RNA samples were analyzed by 10% PAGE (7 M urea). The gels were stained with toluidine blue. (**B**) The RNA samples in panel A were analyzed by northern hybridization. Sequences of the DNA probes for tRNA detection are described in the 'Materials and Methods' section.

Furthermore, the results may explain the discrepancy in the results from experiments concerning the m^5U content. In the nucleoside analysis (Figure 5), the content of the m^5U was decreased in the class I tRNA from the *ΔtrmB* strain. In contrast, the TrmA assay showed that U54 in the *ΔtrmB* strain is near fully modified to m^5U54 or m^5s^2U54 (Figure 6C). U54 hypo-modified tRNA may be preferentially degraded at high temperatures. If so, the degradation of U54 hypo-modified tRNA raises the melting temperature of the class I tRNA fraction. Based on the results from northern hybridization, we concluded that the lack of the m^7G46 and hypo-modifications of

other modified nucleotides cause degradation of some tRNA species ($tRNA^{Phe}$ and $tRNA^{Ile}$) and result in depression of protein synthesis at high temperatures.

DISCUSSION

The m^7G46 modification in tRNA is widely found in eubacteria and eukaryotes. Nevertheless, the role of the m^7G46 modification in eubacterial tRNA has not been clarified: the *E. coli trmB* disruptant showed no growth defect (8). In the current study, we demonstrate the importance of the tRNA m^7G46 modification in the extreme thermophilic eubacterium *T. thermophilus*, which grows at 50–83°C. A summary of our study is shown in Figure 9. In the *ΔtrmB* cultured at high temperatures (above 70°C), several modified nucleotides in tRNA were hypo-modified in addition to the lack of the m^7G46 modification. Although these hypo-modifications do not affect the Phe charging activity of $tRNA^{Phe}$, they cause a decrease in melting temperature and degradation of $tRNA^{Phe}$ and $tRNA^{Ile}$. Protein synthesis of the *ΔtrmB* strain is clearly depressed above 70°C. At 80°C, the *ΔtrmB* strain exhibit a severe growth defect and often shows bacteriolysis during culture. Thus, the m^7G46 modification is essential for cell viability and it may act through a tRNA modification network, in which the m^7G46 modification has a positive effect on other modifications. It was demonstrated that the presence of the m^7G46 in tRNA enhanced Gm18 formation activity by TrmH. Similar results were also obtained with m^1G37 by TrmD and m^1A58 by TrmI, although these modifications were near fully formed in the *ΔtrmB* cells. Thus, these results suggest that TrmB may be one of the key enzymes in the tRNA modification network. It is noteworthy that our preliminary experiment using gel-filtration column chromatography showed no direct interaction between TrmB and TrmH proteins (data not shown), although the source of TrmB was *A. aeolicus*. The m^7G46 modification and stabilized local structure of tRNA seem to have a positive effect on the Gm18 formation by TrmH, although there is a potential for interaction of TrmB and TrmH on the precursor tRNA. Further study will be necessary to clarify the protein–protein and/or protein–tRNA interactions in the network.

For more than 50 years, there has been limited information concerning the role of the m^7G46 modification in tRNA. In the case of anticodon or anticodon loop modifications, lack of the modification often causes disorder of the codon–anticodon interaction and/or frame shift error (43,44). Therefore, at least, lysidine34 (k^2C34) (45), inosine34 (I34) (46) and m^1G37 (47) modifications in *E. coli* are essential for cell viability. In contrast, it is difficult to explain the roles of modified nucleotides in the three-dimensional core of tRNA. In some cases, the disruption of a single three-dimensional (3D) core modification enzyme gene gives no significant apparent phenotype. For example, a null mutant of *E. coli truB*, which encodes tRNA (Ψ55) synthase, grows normally, although the mutant exhibits a defect in survival upon rapid transfer from 37 to 50°C (48). In the case of

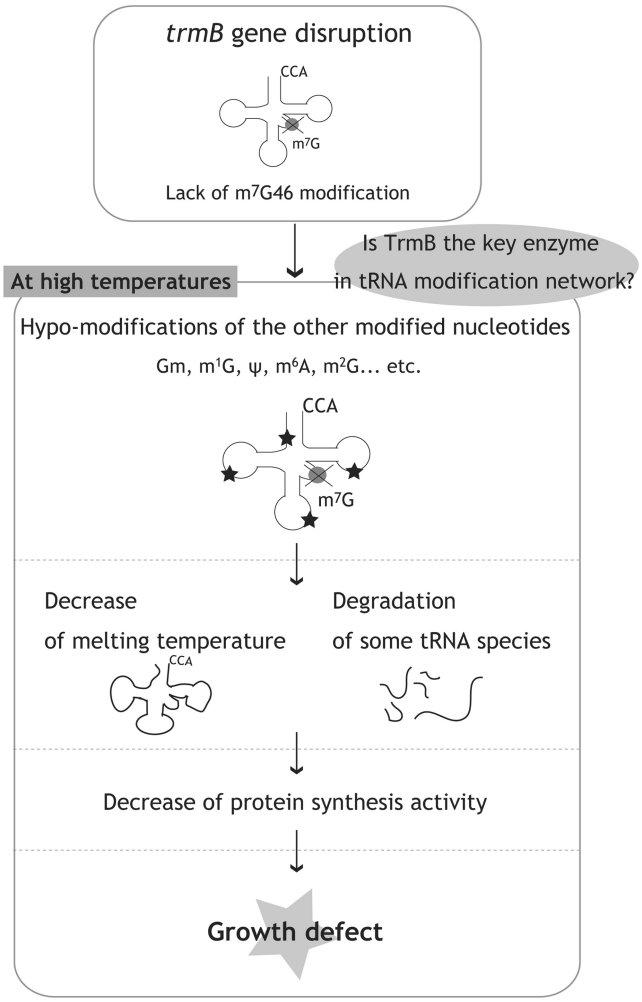

Figure 9. Summary of this study. Effects of the *trmB* gene disruption are depicted. The lack of the m^7G46 modification causes hypo-modification of other nucleotides in class I tRNA. The melting temperature of the tRNA decreases, and $tRNA^{Phe}$ and $tRNA^{Ile}$ are degraded. The degradation of tRNA depresses protein synthesis and the *ΔtrmB* strain exhibits a severe growth defect at high temperatures. These experimental results suggest the existence of a tRNA modification network, in which the m^7G46 modification catalyzed by TrmB may act as one of the key factors.

the *E. coli* m^5U54 modification which is produced by TrmA, the TrmA protein is essential for viability although the known catalytic activity of TrmA is not necessary (33). Because the TrmA protein exists not only in a tRNA bound form but also a 16S rRNA bound form in *E. coli* cells, the TrmA protein seems to have multiple functions (49). Thus, studies of gene disruptant mutants of 3D core modification enzymes are not straightforward. In fact, we have constructed several gene disruptant strains of *T. thermophilus* tRNA modification enzymes and some of them show no apparent change in growth phenotype. For example, a null mutant of *T. thermophilus trmH* grows normally at 70 and 80°C (Iwashita,C. and Hori,H., unpublished results). In contrast, the *ΔtrmB* strain showed a severe growth defect at high temperatures as demonstrated in the current study. Therefore, we consider that the m^7G46 modification may be one of the

key factors in the tRNA modification network. Our findings in the current study may explain the wide distribution of the m^7G46 modification in eubacterial tRNA. Because *T. thermophilus* is an extreme thermophilic eubacterium, we were able to elucidate the importance of the m^7G46 modification at high temperatures, in which unmodified tRNA transcripts are melted. It is likely that primitive life was born on Earth in a high-temperature environment. Thus, RNA modifications in the primitive life are likely to be more important than those in modern mesophiles. In *T. thermophilus*, two modifications, m^5s^2U54 (50) and m^1A58 (31), have been reported to contribute to viability at high temperatures. The m^5s^2U54 modification raises the melting temperature of tRNA (50) and the presence of m^1A58 induces the m^5s^2U54 modification (51). The m^7G46 modification seems to reflect this m^5s^2U54 and m^1A58 modification network because our *in vitro* experiment showed that the m^7G46 modification enhances the speed of m^1A58 formation. However, because the m^1A58 modification in the *ΔtrmB* strain is nearly complete, the modification enzyme for the m^1A58 (TrmI) would seem to be abundant in the cells. The lack of m^7G46 mainly has a positive effect on other modifications such as Gm18 which is catalyzed by TrmH. Although G46 is one of the positive determinants for TrmH as described in our previous report (52), the effect of the G46 modification on TrmH activity has not been elucidated. In *E. coli*, the Gm18 modification contributes to translational accuracy in conjunction with Ψ55 as demonstrated by the fact that a mutant strain lacking both the Gm18 and Ψ55 modifications shows a growth defect and increased frameshift error frequency (53). In the *T. thermophilus ΔtrmB* strain, a similar phenomenon may occur because the level of Gm and Ψ modifications in the *ΔtrmB* cells decreases. In the class I tRNA from *ΔtrmB* cells, we found that the content of the m^1G37 modification is not 100%. The hypo-modification of m^1G37 directly perturbs accuracy of protein synthesis, because this modification prevents frameshift errors (43,54).

Although prevention of tRNA degradation by modified nucleotides is predicted from the results of *in vitro* experiments (55), *in vivo* degradation of the hypo-modified tRNA in bacterial cells has not been experimentally verified. Our experimental results from the current study clearly reveal the degradation of the hypo-modified tRNA in eubacterial cells. Recently, it has been reported that tRNA modifications in yeast function as a quality control system for tRNA. For example, the m^7G46 modification in yeast contributes to the stability of tRNA in conjunction with the other modified nucleotides such as m^5C (16). Furthermore, the m^1A58 modification in yeast is essential for viability through the stability of initiator $tRNA^{Met}$ (56,57). In the *T. thermophilus ΔtrmB* strain, degradation of $tRNA^{Phe}$ and $tRNA^{Ile}$ were observed at high temperatures. Therefore, a primitive quality control system, in which tRNA modifications are monitored, may exist in eubacterial cells. Further study will be necessary to clarify whether a tRNA quality control system in eubacteria exists or not.

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