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

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ABSTRACT

N\textsuperscript{7}-methylguanine at position 46 (m\textsuperscript{7}G46) in tRNA is produced by tRNA (m\textsuperscript{7}G46) methyltransferase (TrmB). To clarify the role of this modification, we made a *trmB* gene disruptant (*D*\textsubscript{trmB}) of *Thermus thermophilus*, an extreme thermophilic eubacterium. The absence of TrmB activity in cell extract from the *D*\textsubscript{trmB} strain and the lack of the m\textsuperscript{7}G46 modification in tRNA\textsuperscript{Phe} were confirmed by enzyme assay, nucleoside analysis and RNA sequencing. When the *D*\textsubscript{trmB} strain was cultured at high temperatures, several modified nucleotides in tRNA were hypo-modified in addition to the lack of the m\textsuperscript{7}G46 modification. Assays with tRNA modification enzymes revealed hypo-modifications of G\textsubscript{m18} and m\textsuperscript{1}G\textsubscript{37}, suggesting that the m\textsuperscript{7}G46 positively affects their formations. Although the lack of the m\textsuperscript{7}G46 modification and the hypo-modifications do not affect the Phe charging activity of tRNA\textsuperscript{Phe}, they cause a decrease in melting temperature of class I tRNA and degradation of tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Ile}. \textsuperscript{35}S-Met incorporation into proteins revealed that protein synthesis in *D*\textsubscript{trmB} cells is depressed above 70°C. At 80°C, the *D*\textsubscript{trmB} strain exhibits a severe growth defect. Thus, the m\textsuperscript{7}G46 modification is required for cell viability at high temperatures via a tRNA modification network, in which the m\textsuperscript{7}G46 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\textsuperscript{7}-methylguanine at position 46 (m\textsuperscript{7}G46) in the variable region is one of the common modifications in tRNA and forms a tertiary base pair with the C\textsubscript{13}–G\textsubscript{22} base pair (4,5).

The m\textsuperscript{7}G46 modification is generated by tRNA (m\textsuperscript{7}G46) methyltransferase [tRNA (guanine-N\textsuperscript{7}-)methyltransferase, EC 2.1.1.33; TrMet (m\textsuperscript{7}G46)] (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\textsuperscript{7}G46) 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...
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’G46 modification have also recently been performed. Gene disruption mutants of yeast (7) or E. coli (8) have revealed that the tRNA (m’G46) 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 trm8 and trm4 genes showed rapid degradation of tRNAVal (16) [trm4 encodes yeast tRNA (m’C34, 40, 48, 49) methyltransferase (17)]. Thus, the m’G46 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’G 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 (AtmB) strain of Thermus thermophilus HB8, an extreme thermophilic eubacterium. We report the importance of the m’G46 modification for growth at high temperatures and propose a tRNA modification network, in which the m’G46 modification has a positive effect on formation of other modifications in tRNA.

**MATERIALS AND METHODS**

**Materials**

[Methyl-14C]-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 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 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 GTG 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 AtmB (ΔTTHA1619) 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 ΔTTHA1619 were prepared from late-log phase cells cultivated at 67°C for
in vitro methyl-transfer assay. Wet cells (0.3 g) were suspended in 2 ml of buffer A [50 mM Tris–HCl (pH 7.6), 5 mM MgCl₂, 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 A₂₆₀ unit yeast tRNA<sub>Phe</sub> transcript and 0.78 nmol [methyl<sup>14</sup>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 <sup>14</sup>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 ΔTTHA1619 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 MgCl₂, 400 mM NaCl] and then a small RNA fraction (mainly tRNA and SS 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 A₂₆₀ 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 x 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<sup>1</sup>A), 5-methylcytidine (m<sup>5</sup>C), 2'-O-methyladenosine (Am), 2'-O-methylcytidine (Cm) and N<sup>6</sup>-methyladenosine-5'-monophosphate (pm<sup>6</sup>A)] were purchased from Sigma. The pm<sup>6</sup>A nucleotide was dephosphorylated with bacterial alkaline phosphatase before use. 5-Methyl-2-thiouridine (m<sup>2</sup>s<sup>2</sup>U) 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<sup>1</sup>G), 2-methylguanosine (m<sup>2</sup>G), 2'-O-methylguanosine (Gm), 7-methylguanosine (m<sup>7</sup>G), 5-methyluridine (m<sup>5</sup>U) 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<sub>Phe</sub> 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<sub>Phe</sub>. Purification of tRNA<sub>Phe</sub> by solid-phase DNA probe was performed as described in our previous report (25). The eluted tRNA<sub>Phe</sub> was further purified by 10% PAGE (7 M urea).

RNA sequencing

RNA sequences of the purified tRNA<sub>Phe</sub> (0.02 A₂₆₀ unit) from the wild-type and ΔTTHA1619 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<sub>Phe</sub> 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'-<sup>32</sup>P-labeled nucleotides were monitored with a Fuji Photo Film BAS2000 imaging analyzer. Standard nucleotides were marked by UV<sub>260nm</sub> 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<sub>600nm</sub> = 0.1) and sequential dilutions (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup>) (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 <sup>35</sup>S-Met incorporation

The wild-type and ΔtrmB strains were cultured at 70°C. When the cell density (A<sub>600nm</sub>) 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 <sup>35</sup>S-Met. Culture medium (1 ml each) was sampled at various timepoints and the cells collected by centrifugation at
3500g for 2 min. The cells were washed with 500 µl of the medium and collected by centrifugation at 3500g 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 500g for 5 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.

**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^4G64 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 MgCl2, 100 mM NaCl] from 80 to 40°C 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* (rRNA (Gm18) methyltransferase (TrmH) (23,27–30) and tRNA (m^1A58) methyltransferase (TrmI) (31) were kindly provided by Anna Ochi (Ehime University) and Masayuki Minoji (Ehime University), respectively. *Escherichia coli* tRNA m^3U54 methyltransferase (TrmA) (32–34) was a gift from Chikako Iwashita (Ehime University). *Aquifex aeolicus* tRNA (m^1G37) 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 KC1 linear gradient (50–350 mM). The fractions containing Phe-RS were identified by Phe charging activity. The aminoacylation assay was performed using 14C-Phe (18.4 GBq/mmol, PerkinElmer) as described in the reference (38). Phe charging activity was measured using 0.03A260 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'-32P-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 GCA TGC TCT ACC AA-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^Val^ (UAC), 5'-ATC AGG CGT GCC 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^7G46 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 14C-methyl transfer assay using yeast and *T. thermophilus* tRNA^Phe^ transcripts, and 14C-pmG 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 ΔTTHA1619 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 ΔTTHA1619 strain.

**Absence of tRNA (m^7G) methyltransferase activity in cell extract from the ΔTTHA1619 strain**

To confirm the absence of tRNA (m^7G) methyltransferase activity in the ΔTTHA1619 strain, we prepared S-100 and P-100 wash fractions from the wild-type and ΔTTHA1619 strains. The S-100 fraction from the wild-type or ΔTTHA1619 strain, yeast tRNA^Phe^ transcript, and 14C-AdoMet were incubated at 60°C for 1 h. The RNA
was recovered by ethanol precipitation and digested with nuclease P1. The 14C-labeled mononucleotides were analyzed by 2D-TLC. Positions of standard markers (pA, pG, pC and pU) are enclosed by dotted circles. The nucleoside analyses of the class I tRNA fractions from the wild-type (upper) and ΔTTHA1619 (lower) strains. 0.03 A260 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).

Figure 1. Absence of m7G formation activity and lack of m7G nucleoside in extract and tRNA from the ΔTTHA1619 strain. (A) Yeast tRNA\textsuperscript{Phe} transcript, [methyl-\textsuperscript{14}C]-AdoMet and S-100 fraction of the wild-type (left) or ΔTTHA1619 (right) strain were incubated at 60°C for 1 h, and 14C-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 ΔTTHA1619 (lower) strains. 0.03 A\textsubscript{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).
experimental results together, we concluded that the tRNA (m^7G) methyltransferase activity was absent from the cell extract of the ΔTTHA1619 strain.

Absence of the m^7G modification in the Class I tRNA fraction from the ΔTTHA1619 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 ΔTTHA1619 strains were purified as shown in Figure 1B inset. 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 ΔTTHA1619 sample (Figure 1B, lower). Thus, we confirmed the absence of the m^7G46 modification in class I tRNA from the ΔTTHA1619 strain.

As well as the alteration described above we also noticed changes in other modifications of class I tRNA from the ΔTTHA1619 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^1Atom^6A. Another is the m^6A37 modification, for which the responsible enzyme has not been identified. Moreover, at least, three modifications (Gm18, m^5s2U54 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

Figure 2. Sequence analyses of purified tRNA^{Phe} from the wild-type and ΔTTHA1619 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 ΔTTHA1619 (right) strains were analyzed by 10% PAGE (7M urea). The gel was stained by toluidine blue. The purified tRNA^{Phe} from the wild-type (C) and ΔTTHA1619 (G) strains was partially cleaved by formamide. Then the 5’-end of each fragment was labeled with γ-32P-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 ΔTTHA1619 (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.
modification in tRNA\textsuperscript{Phe} from the \textit{ATTHA1619} strain, we purified tRNA\textsuperscript{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\textsuperscript{Phe} is quite different from those of the other \textit{T. thermophilus} tRNA species. To elute the RNA efficiently, tetracylammonium chloride was selected as the salt in the hybridization buffer. This approach was successful in purifying tRNA\textsuperscript{Phe} from tRNA mixtures of the wild-type and \textit{TTHA1619} strains (Figure 2B). We further purified the tRNA\textsuperscript{Phe} by 10\% PAGE (7 M urea).

To determine precise positions of the modified nucleosides, we performed RNA sequencing. In these experiments, we selected Kucchin's post label method (26) to visualize the modifications. Briefly, the purified tRNA\textsuperscript{Phe} was partially cleaved by formamide, and then the 5'-end of each fragment was labeled with \textit{\gamma}-\textit{32P}-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 32P-labeled nucleotides were analyzed by TLC. Because the RNA sequence of \textit{T. thermophilus} is quite different from those described here may be caused by differences in the m\textsuperscript{5}G46 modification between previous studies and our results, the TTHA1619 gene is \textit{trmB}

35S-Met incorporation into proteins in the \textit{Atmb} strain decreased not only at 80\°C but also at 70\°C

We compared \textit{in vivo} protein synthesis activities of the wild-type and \textit{Atmb} strains by monitoring 35S-Met incorporation. When the cell density had reached 0.5Al\textsubscript{600} nm, 35S-Met was directly added into the culture medium. Figure 4A shows the results at 70\°C. Unexpectedly, the speed of 35S-Met incorporation into proteins in the \textit{Atmb} 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 \textit{Atmb} 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, 35S-Met incorporation of the \textit{Atmb} 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\textsuperscript{7}G46 modification in tRNA causes depression of protein synthesis at high temperatures.

Other modifications are decreased in the class I tRNA fraction from the \textit{Atmb} cells cultured at 70–80\°C

We have observed decreases in some modifications in tRNA from the \textit{Atmb} strain at 70\°C as described
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^3U, 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
affects the activities of several tRNA modification enzymes. To confirm this idea, we performed assays using tRNA modification enzymes. The Gm18, m^{1}G37, m^{5}U54 and m^{1}A58 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
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^5G46 modification, we modified total RNA from the ΔtrmB strain with A. aeolicus TrmB (we prepared the total RNA from the ΔtrmB strain with m^5G46 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 ^14C-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^5G46 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^1U54 or m^5s2U54 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^G37 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^A58 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^G37 by TrmD and m^A58 by TrmI. The lack of the m^7G46 modification causes hypo-modifications of Gm18 and m^G37 (m^A58 is slightly hypo-modified). In the nucleoside analysis (Figure 5B), the contents of Ψ, m^2G, m^6A and m^G + Gm decreased. The Ψ modifications are introduced into various positions by multiple enzymes. The enzymes responsible for the m^2G6 and m^A37 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
the ΔtrmB strain at 70 and 80°C. Furthermore, we examined Phe charging activity of the tRNA\textsuperscript{Phe} from the wild-type and 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\textsuperscript{7}G46 and the presence of hypo-modifications do not affect the Phe charging activity of tRNA\textsuperscript{Phe}.

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

The lack of m\textsuperscript{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\textsuperscript{7}G46 modification to melting temperature, we prepared yeast tRNA\textsuperscript{Phe} transcript containing m\textsuperscript{7}G46 by \textit{in vitro} methyl-transfer reaction with \textit{A. aeolicus} TrmB. As shown in Table 1, the presence of the m\textsuperscript{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\textsuperscript{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
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’G46 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.

Furthermore, the results may explain the discrepancy in the results from experiments concerning the m’U content. In the nucleoside analysis (Figure 5), the content of the m’U 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’5U or m’5s2U (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’G46 and hypo-modifications of
other modified nucleotides cause degradation of some tRNA species (tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Ile}) and result in depression of protein synthesis at high temperatures.

**DISCUSSION**

The m\textsuperscript{7}G46 modification in tRNA is widely found in eubacteria and eukaryotes. Nevertheless, the role of the m\textsuperscript{7}G46 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\textsuperscript{7}G46 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\textsuperscript{7}G46 modification. Although these hypo-modifications do not affect the Phe charging activity of tRNA\textsuperscript{Phe}, they cause a decrease in melting temperature and degradation of tRNA\textsuperscript{Phe} and tRNA\textsuperscript{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\textsuperscript{7}G46 modification is essential for cell viability and it may act through a tRNA modification network, in which the m\textsuperscript{7}G46 modification has a positive effect on other modifications. It was demonstrated that the presence of the m\textsuperscript{7}G46 in tRNA enhanced Gm18 formation activity by TrmH. Similar results were also obtained with m\textsuperscript{1}G37 by TrmD and m\textsuperscript{1}A58 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\textsuperscript{7}G46 modification and stabilized local structure of tRNA seem to have a positive effect on the Gm18 formation by TrmH. Similar results were also obtained with m\textsuperscript{1}G37 by TrmD and m\textsuperscript{1}A58 by TrmI, although these modifications were near fully formed in the *ΔtrmB* cells. Further study will be necessary to clarify the protein–tRNA interactions in the network.

For more than 50 years, there has been limited information concerning the role of the m\textsuperscript{7}G46 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\textsuperscript{2}C34) (45), inosine34 (I34) (46) and m\textsuperscript{1}G37 (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 the *E. coli* m\textsuperscript{5}U54 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\textsuperscript{7}G46 modification may be one of the

![Figure 9. Summary of this study. Effects of the *trmB* gene disruption are depicted. The lack of the m\textsuperscript{7}G46 modification causes hypo-modification of other nucleotides in class I tRNA. The melting temperature of the tRNA decreases, and tRNA\textsuperscript{Phe} and tRNA\textsuperscript{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\textsuperscript{7}G46 modification catalyzed by TrmB may act as one of the key factors.](image)
key factors in the tRNA modification network. Our findings in the current study may explain the wide distribution of the m'G46 modification in eubacterial tRNA. Because *T. thermophilus* is an extreme thermophilic eubacterium, we were able to elucidate the importance of the m'G46 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's^2^U54 (50) and m'\A^58 (31), have been reported to contribute to viability at high temperatures. The m's^2^U54 modification raises the melting temperature of tRNA (50) and the presence of m'\A^58 induces the m^3^s^2^U54 modification (51). The m'G46 modification seems to reflect this m's^2^U54 and m'\A^58 modification network because our *in vitro* experiment showed that the m'G46 modification enhances the speed of m'\A^58 formation. However, the m'\A^58 modification in the *AtrmB* strain is nearly complete, the modification enzyme for the m'\A^58 (TrmI) would seem to be abundant in the cells. The lack of m'G46 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 \Psi^55 as demonstrated by the fact that a mutant strain lacking both the Gm18 and \Psi^55 modifications shows a growth defect and increased frameshift error frequency (53). In the *T. thermophilus* *AtrmB* strain, a similar phenomenon may occur because the level of Gm and \Psi modifications in the *AtrmB* cells decreases. In the class I tRNA from *AtrmB* cells, we found that the content of the m'G37 modification is not 100%. The hypo-modification of m'G37 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'G46 modification in yeast contributes to the stability of tRNA in conjunction with the other modified nucleotides such as m'\C^16 (16). Furthermore, the m'\A^58 modification in yeast is essential for viability through the stability of initiator tRNA^Met^ (56,57). In the *T. thermophilus* *AtrmB* strain, degradation of tRNA^Phe^ and tRNA^Leu^ 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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