Temperature-dependent Biosynthesis of 2-Thioribothymidine of *Thermus thermophilus* tRNA

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2-Thioribothymidine (s\(^2\)T) is a modified nucleoside of U, specifically found at position 54 of tRNAs from extreme thermophilic microorganisms. The function of the 2-thiocarbonyl group of s\(^2\)T54 is thermostabilization of the three-dimensional structure of tRNA; however, its biosynthesis has not been clarified until now. Using an *in vivo* tRNA labeling experiment, we demonstrate that the sulfur atom of s\(^2\)T in tRNA is derived from cysteine or sulfate. We attempted to reconstitute 2-thiolation of s\(^2\)T in *vitro*, using a cell extract of *Thermus thermophilus*. Specific 2-thiolation of ribothymidine, at position 54, was observed *in vitro*, in the presence of ATP. Using this assay, we found a strong temperature dependence of the 2-thiolation reaction *in vitro* as well as expression of 2-thiolation enzymes *in vivo*. These results suggest that the variable content of s\(^2\)T *in vivo* at different temperatures may be explained by the above characteristics of the enzymes responsible for the 2-thiolation reaction. Furthermore, we found that another posttranscriptionally modified nucleoside, 1-methyladenosine at position 58, is required for the efficient 2-thiolation of ribothymidine 54 both *in vivo* and *in vitro*.

Post-transcriptional modification is a characteristic feature of RNA molecules. In transfer RNA, post-transcriptional modification plays various roles that are required for the translation process, including fidelity control of codon recognition, reading frame maintenance, and stabilization of the tertiary tRNA structure (1). 2-thioribothymidine (s\(^2\)T) is a 2-thiolated derivative of 5-methyluridine (ribothymidine (rT)), located at position 54 of *Thermus thermophilus* tRNA (2), and has been shown to stabilize tRNA structure in a high temperature environment (3). In the extreme thermophilic eubacteria, *Thermus thermophilus* (4), and the archaea, *Pyrococcus furiosus* (5), the 2-thiolation level of rT54 in tRNA increases with cultivation temperature; the melting temperature (T\(_m\)) of the tRNA increases concomitantly with incremental increases in s\(^2\)T content. These findings indicate that 2-thiolation of rT54 is responsible for the thermostability of thermophile tRNA at a variety of cultivation temperatures, thereby ensuring the adaptation of the protein synthesis machinery to specific environmental conditions.

However, the body of knowledge regarding s\(^2\)T biosynthesis is limited. First, the enzymes responsible for the tRNA modification and the genes involved in the 2-thiolation reaction have not been identified to date. Previously, we demonstrated that the tRNA structural elements required for 2-thiolation are the conserved bases in the T\(_4\)C loop and the structure created by those bases (6). In this report, we investigated the sulfur donor for s\(^2\)T biosynthesis *in vivo*. We also examined the 2-thiolation reaction *in vitro*, in order to characterize the temperature dependence of this reaction, and considered the contribution of neighboring nucleoside modifications to the efficiency of 2-thiolation of rT54, both *in vivo* and *in vitro*.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—*T. thermophilus* HB8 was used as the wild-type strain throughout this study. *Escherichia coli* JM109 was used as the bacterial host for the genetic manipulation of plasmids. Other strains used in this study are listed in Table 1. Rich medium and minimal medium (MM) for *T. thermophilus* were used as described previously (9). Both wild-type and mutant cells were cultivated in the rich medium without or with 30 µg/ml kanamycin, respectively, unless otherwise stated. For *in vivo* labeling of RNA, minimal medium without sulfur compounds (MM−S) was prepared. The salts used had chloride ions (Cl\(^−\)) substituted for sulfate ions (SO\(_4^{2−}\)) as follows: (NH\(_4\))\(_2\)SO\(_4\) to NH\(_4\)Cl, ZnSO\(_4\)-7H\(_2\)O to ZnCl\(_2\), CuSO\(_4\)-5H\(_2\)O to CuCl\(_2\), and FeSO\(_4\)-7H\(_2\)O to FeCl\(_3\)-6H\(_2\)O. VOSO\(_4\)-xH\(_2\)O, biotin, and thiamine-HCl were not used as supplements in MM−S medium.

**In Vivo Labeling of RNA**—Cells were cultivated overnight in 10 ml of MM at 70 °C, centrifuged, and washed with MM−S medium. They were then incubated with shaking at 70 °C for 1 h in 10 ml of MM−S supplemented with 0.27 mCi of [\(^{35}\)S]methionine, 0.41 mM cysteine, and 0.4 mM sodium sulfate for the methionine labeling; 0.27 mCi of [\(^{35}\)S]cysteine, 0.33 mM methionine, and 0.4 mM sodium sulfate for the cysteine labeling; and 0.27 mCi of [\(^{35}\)S]sulfate for the sulfate labeling. [\(^{35}\)S]-Labeled compounds were purchased from American Radiolabeled Chemicals. Total RNA was extracted from the cells using ISOGEN (Wako). For alkaline treatment of tRNA, total RNA was incubated at 37 °C for 1 h in 100 mM HEPES-KOH (pH 9.0). Then total RNA was separated by 10% PAGE containing 7 M urea, and the gel was dried and exposed to an imaging plate, followed by analysis using a BAS5000 bioimaging analyzer (Fuji Photo Systems).
Using the plasmids and fragments prepared as described above, T. thermophilus HB8 was transformed according to the literature (12) and then selected on a rich plate containing 300 μg/ml kanamycin at 70 °C. Site-specific homologous recombination of transformants was confirmed by PCR amplification, followed by restriction enzyme digestion or Southern hybridization.

**Modified Base Analysis of Total tRNA by LC/MS—**Cells were cultivated at 70 °C to late log phase. Total RNAs were extracted from cells using Isogen (Wako), and the tRNA fraction was further purified by 10% PAGE containing 7 M urea. Nucleoside analysis of total tRNA was performed by LC/MS as described previously (10). The s2T content was calculated from the area under the UV peak and normalized by the peak of pseudouridine (ψ) in each data set.

**In Vitro Thiolation Assay—**s3T-deficient tRNA<sup>Bio</sup> (Kenjo Miyautchi, University of Tokyo) was prepared from the E. coli JD20605 strain (Dr. Miki Takeyosi, Fukuoka Dental College). tRNA<sup>Bio</sup> from Saccharomyces cerevisiae was purchased from Sigma. tRNA<sup>Bio</sup> with 1-methyladenosine (m<sup>1</sup>A) S8 was prepared by recombinant T. thermophilus Trm1 protein, which was expressed in the E. coli strain Rosetta (DE3) (Nagaven), with plasmid pML24, and purified as described previously (13). tRNA<sup>Bio</sup> from the E. coli thiI mutant (8 μg) was methylated at 60 °C for 1 h in a reaction mixture containing 50 mM Tris–HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 50 μM S-adenosylmethionine (Sigma), and 2 μg of Trm1. The reaction was stopped by phenol extraction, and the tRNA was ethanol-precipitated.

Unmodified tRNA<sup>Bio</sup> (with a C1G mutation) and tRNA<sup>Bio</sup> (with C1G/U54A mutations) were prepared by in vitro transcription using T7 RNA polymerase (14). Template for in vitro transcription was constructed by PCR using synthetic oligonucleotide DNAs carrying the tRNA gene under the T7 promoter sequence (14). Oligonucleotide DNAs used for the construction of a plasmid-bearing gene for tRNA<sup>Bio</sup> are Met–C1G-F (ggccGAATTC ttaacctcctagag cccggg gctggtgag) and Met–C1G-R (ccgccAAGCT gtcgctgctc ctcggcg). The PCR products were cloned into the EcoRI and HindIII sites of pUC19. For in vitro transcription, the pair of primers Met–F (ttaacctcctagag) and Met–R (ctcgctgctc ctcggcg) was employed to PCR-amplify the template plasmid pUC19–tRNA<sup>Bio</sup>C1G, respectively. Transcripts of tRNA genes were prepared at 37 °C for 3 h in a reaction mixture containing 40 mM HEPES–KOH (pH 7.8), 5 mM dithiothreitol, 1 mM spermidine, 8 mM MgCl<sub>2</sub>, 1 mM each NTPs, 5 mM GMP, 50 μg/ml bovine serum albumin, 2 μg/ml template DNA, and T7 RNA polymerase (14). Then the reaction for CCA addition was performed as described (15).

tRNAs were dephosphorylated using bacterial alkaline phosphatase (Takara) and labeled at their 5′ termini with [γ-<sup>32</sup>P]ATP (110 TBq/
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**TABLE 2**

Oligonucleotide DNAs used in gene disruptions in this study

| Primers | Sequences | Amplification regions |
|---------|------------|-----------------------|
| For NS0801 | 5’-cttgggtgccg tggggcctct-3’ | biotin and flanking regions |
| thil-F | 5’-cttgggtgccg tggggcctct-3’ | biotin and flanking regions |
| thil-R | 5’-cttgggtgccg tggggcctct-3’ | biotin and flanking regions |
| htk-Ncol-F | 5’-atacgaCTAG Ggtagggcc gattagcta c-3’ | htk |
| htk-Ncol-R | 5’-atacgaCTAG Ggtagggcc gattagcta c-3’ | htk |

mmol, Amersham Biosciences) and T4 polynucleotide kinase (Toyobo) and purified using denaturing 10% PAGE.

Cell extract used for the *in vitro* assay was prepared from early log phase cells that had been cultured at either 80 or 50 °C. Cells were resuspended with standard buffer containing 50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl2, and 0.2 mM phenylmethylsulfonyl fluoride. Resuspended cells were sonicated at 4 °C and then centrifuged at 20,000 × g for 15 min at 4 °C. The supernatant (S20) was used as “lysate.” Additionally, we performed a 100,000 g centrifugation (S100) followed by gel filtration of the supernatant with a Nap5 column (Amersham Biosciences), to prepare the lysate for the ms2i6A dependence assay (Fig. 5C).

Standard reactions were performed with 5'-labeled tRNA (30,000 – 50,000 cpm), 5 mM ATP, and lysate (10 – 15 mg/ml) in 20 μl of standard buffer at 50 °C for 20 min, unless otherwise indicated. tRNA was recovered using Isogen (Wako), precipitated with ethanol, and then electrophoresed on [(N-acryloylaminophenyl)mercuric chloride (APM) gels, 7 M urea. The recovered tRNA in 3'-fragments were analyzed by APM-PAGE.

**Temperature Sensitivity Assay**—Cells were cultivated overnight at 70 °C. Cultures diluted to 10^-³, 10^-⁴, or 10^-⁵ were spotted onto rich plates and incubated for 32 h at 70 or 80 °C, respectively.

**Transfer RNA Melting Analysis**—Melting curves for tRNAs were measured in 50 mM sodium cacodylate buffer, pH 7.5, in the presence of 10 mM MgCl2 and 200 mM NaCl. tRNA hyperchromicities at 260 nm were recorded using a spectrophotometer, RESPONSE II (Gilford).

**RESULTS**

Cysteine and Sulfate Ion Are the Sulfur Donors for s^2T Biosynthesis in Vivo—To identify the sulfur donor for s^2T modification, we performed an *in vivo* labeling experiment. It is known that cysteine is a sulfur donor for tRNA modifications, such as 4-thiouridine (s4U), 5-methylamino-methyl-2-thioribofuranoside (mms^2A), and 2-thiocytidine (s2C), in *E. coli* (20). Also in *Salmonella typhimurium*, cysteine is a sulfur donor for tRNA modifications as above, and instead of mms^2A, tRNA contains 6-(4-hydroxysopentanyl)-2-methylthioadenosine (ms^2io^6A) (21). In light of this knowledge, we tested the possibility that cysteine and other sulfur compounds are incorporated into the s^2T of *T. thermophilus* tRNA.

s^2T tRNAs were cultivated for 1 h in minimal medium containing s^2S-labeled methionine, cysteine, or sodium sulfate. Total RNA was extracted from the cells and analyzed by denaturing PAGE. A [^35S]methionine-labeled band appeared in one tRNA species, and the band disappeared with mild alkaline treatment of the sample (Fig. 1A, lanes 1 and 2). This indicates that the band was probably derived from [^35S]methionyl-tRNA. When grown in the presence of labeled cysteine or sulfate ions, bands corresponding to all of the tRNA species were uniformly labeled with ^35S and were unaffected by mild alkaline treatment (Fig. 1A, lanes 3–6). These bands are believed to be derived from tRNAs modified with ^35S.

*T. thermophilus* tRNAs possess s^U at position 8, one of the major sulfur-modified nucleosides together with s^T (4). Therefore, labeled RNA was digested into nucleosides and analyzed by an ODS reversed-phase column, in order to confirm that the sulfur atom of cysteine and...
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We constructed two mutant strains of \textit{iscS} or \textit{sufS} by homologous recombination and confirmed the genomic organization around the target genes by Southern hybridization or PCR (data not shown). Formation of s\textsuperscript{2}T in tRNA was not affected in these mutant strains, as determined by nucleoside analyses of total tRNA with reversed-phase HPLC, coupled with a mass spectrometer. s\textsuperscript{2}T was detected as the protonated form \((m/z = 275)\) and protonated free-base form \((m/z = 143)\). The s\textsuperscript{2}T contents of \textit{iscS} and \textit{sufS} mutants were 1.2 and 0.9, when compared with that of the wild type (set at 1), respectively. These results suggest redundant roles for IscS and SufS in the synthesis of s\textsuperscript{2}T. However, it is possible that another cysteine-utilizing enzyme, distinct from IscS and SufS, is present in \textit{T. thermophilus}.

Detection of 2-Thiolation Activity in a \textit{T. thermophilus} Cell Extract—To characterize the 2-thiouridylation reaction in detail, we tried to detect its activity \textit{in vitro}. We used the affinity electrophoresis method (APM gel electrophoresis) developed originally by Igloi (16), since we have succeeded previously in the detection of the 2-thiolation of U54 using this method (6). First, we used tRNA\textsuperscript{Met} from the \textit{E. coli} thiil mutant strain as the substrate, since the nucleotide sequence of the tRNA is almost identical to that of tRNA\textsuperscript{Met} from \textit{T. thermophilus}. At the unmodified nucleoside level, the differences are that the U50 of \textit{E. coli} tRNA is C50 in \textit{T. thermophilus} (25) (Fig. 2A) and that the tRNA from the thiil mutant has no s\textsuperscript{4}U (26). s\textsuperscript{4}U is known to be retarded greatly in APM gels (16).

In addition, we constructed an s\textsuperscript{4}U-deficient strain of \textit{T. thermophilus} (thiil mutant). The gene thiil encodes a thiotransferase that is required for s\textsuperscript{4}U8 synthesis in \textit{E. coli} (26). We constructed a thiil mutant of \textit{T. thermophilus} (NS0801), and tRNA recovered from the mutant cells was analyzed by LC/MS. The tRNA modification of NS0801 was the same as wild type, with the exception of the complete loss of s\textsuperscript{4}U (data not shown). This indicates that thiil of \textit{T. thermophilus} encodes a genuine thiotransferase for s\textsuperscript{4}U8 synthesis.

Using the s\textsuperscript{4}U-deficient, native tRNA\textsuperscript{Met} from \textit{E. coli} as the substrate, ATP-dependent 2-thiouridylation was detected, suggesting the formation of s\textsuperscript{2}T (Fig. 2B). When the wild-type lysate was used, migrating bands corresponding to both 4-thiouridyalted tRNA (greatly retarded) and 2-thiouridyalted tRNA (slightly retarded) were detected (lane 3), but only a single retarded band, corresponding to 2-thiouridyalted tRNA, was detected when lysate from the mutant NS0801 was used (lane 5).

To confirm that the 2-thiouridylation occurred at position 54 in the tRNA, we performed the same experiment using tRNA transcripts (Fig. 2A), in which C1 was substituted by G1, to obtain more efficient transcription in \textit{vitro} (14). The tRNAs recovered from the reaction mixture were analyzed by APM gel electrophoresis, by which 2- and 4-thiouridylations of tRNA\textsuperscript{Met} transcript of the U54A mutant were examined as compared with those of the wild type (Fig. 2B). The wild-type tRNA transcript was thiolated at a slightly lower level than the native tRNA\textsuperscript{Met} (compare lane 5 in Fig. 2B, a and b), and this may be caused by the presence of other modified nucleosides in the native tRNA. The U54A mutant was effectively 4-thiouridyalted (Fig. 2B, lane 8) but not 2-thiouridyalted (Fig. 2B, lane 10), suggesting that 2-thiouridylation occurred at position 54.

In order to analyze the modified nucleosides, we performed RNA sequencing of the tRNA\textsuperscript{Met} from the \textit{E. coli} thiil mutant, reacted \textit{in vitro} with the lysate from NS0801 (thiil::km). First, we separated the reacted tRNA into the upper (retarded) and lower (not retarded) bands using a preparative scale APM gel electrophoresis, like the electrophoretic pattern shown in lane 5 of Fig. 2B. The bands were then excised, recovered from the gel, and subjected to RNA sequencing. The ladder pat-

the sulfate ion were actually incorporated into s\textsuperscript{2}T. Two major \textsuperscript{35}S-labeled peaks were detected in the RNA hydrolysate (Fig. 1B). These were identified as s\textsuperscript{4}U and s\textsuperscript{2}T by comparing their elution times with those of authentic nucleosides. The chromatogram pattern was essentially the same for both \textsuperscript{35}S-sulfate and \textsuperscript{35}S-cysteine labeling (data not shown). These results clearly demonstrate that the sulfur atoms from cysteine and the sulfate ion were incorporated into the s\textsuperscript{2}T of tRNA \textit{in vivo}.

\textit{iscS} and \textit{sufS} Mutations Did Not Affect s\textsuperscript{2}T Biosynthesis in \textit{T. thermophilus}—IscS (iron sulfur cluster) is the cysteine desulfurase that is responsible for all of the thio-modifications in \textit{E. coli} (20) and \textit{S. typhimurium} (21). This enzyme catalyzes the initial sulfur transfer reaction from cysteine. It is believed that, in \textit{T. thermophilus} (see above), the sulfur atom from cysteine is also incorporated into s\textsuperscript{2}T, so that an IscS homologue in \textit{T. thermophilus} may be a candidate enzyme for s\textsuperscript{2}T biosynthesis. A genomic database search of \textit{T. thermophilus} HB8 revealed that there are two putative cysteine desulfurase-encoding genes. Alignment of the deduced sequence of these enzymes with the sequence of \textit{E. coli} IscS and SufS (named after the mobilization of sulfur), strongly suggests that the two cysteine desulfurase-like proteins of \textit{T. thermophilus} are IscS and SufS homologues (data not shown). The difference between the two enzymes is indicated by the motif around a conserved catalytic cysteine; a consensus sequence (SSGSACTS) around Cys\textsuperscript{328} of \textit{E. coli} IscS can effectively distinguish this enzyme from SufS, which has a consensus sequence of RXGHHC2A (22). Both IscS and SufS are known to be involved in Fe–S cluster assembly, together with other components of the ISC and SUF machinery (23, 24). However, only IscS (not SufS) is involved in tRNA thiolation in \textit{E. coli} (20).
terns of the lower and upper band samples were almost identical, except for the A in position 58 (see below) of a normal sequencing gel (Fig. 3A). When analyzed using the APM-containing sequencing gel (Fig. 3B), the ladder pattern of the lower band sample was essentially the same as that found using a normal gel (Fig. 3A). Although the ladders below G53 of the upper band sample electrophoresed in a similar manner for both the normal and APM gels, the ladders above position 54 disappeared through upper shifting in the APM gel (shown by the arrow in Fig. 3B), suggesting the presence of a thiocarbonyl nucleoside at position 54, probably attaching at position 2 of the uridine base.

At position 58 (Fig. 3A, asterisk), the tRNA of the upper band was not digested by RNase U2 (compare this with the position of the tRNA in the lower band), suggesting that the upper band possesses m1A58. Thus, the tRNA of the upper band possesses both s2T54 and m1A58, but the tRNA of the lower band possesses rT54 and unmodified A58. During the reaction, the methyl moiety is thought to be incorporated into A58 by a specific methyltransferase included in the lysate. This result suggests that methylation of A58 is required for s2T biosynthesis (see below).

Temperature Dependence of the 2-Thiouridylation Reaction—The s2T content of thermophile tRNA is known to vary depending on the cultivation temperature (4, 5), but the mechanism underlying this regulation is still unknown. In particular, we investigated the question of whether or not either the abundance or activity of the 2-thiolation enzyme(s) is temperature-regulated. We analyzed the temperature

![Diagram](image-url)
dependence of 2-thiouridylation activity in vitro. Since s^2U8 content is known to be both constant and independent of cultivation temperature (4), we analyzed 4-thiouridylation activity as a control. To estimate the 2-thiolation of rT54, we used lysate from the mutant NS0801 (thiI::km) and tRNAPhe from S. cerevisiae, which contains m1A58 (25). Thus, any effects caused by methylation of A58 could be disregarded. We used lysate from HB8 and a transcript of tRNAfMet possessing C1G and U54A mutations for estimation of 4-thiolation of U8.

Using these reactions, the thiolation activity for tRNA was measured by APM gel electrophoresis (Fig. 4A (a and b)), and the band intensities were quantified (Fig. 4A (c)). The substrate tRNAs were greatly degraded above 70 °C, and this precluded the measurement of thiolation activity above that temperature. The 2-thiolation activity of rT54 was very low at 50 °C but increased with temperature, rising 7-fold at 70 °C. However, the 4-thiolation activity increased by only 2-fold throughout the same temperature range.

We measured the enzymatic activities of the lysate obtained from the cells cultured at 50 and 80 °C (Fig. 4B). The 4-thiolation activity of the lysate from the cells grown at 80 °C was almost the same as that of the 50 °C lysate, whereas the 2-thiolation activity of the 80 °C lysate was 13-fold higher than that of the 50 °C lysate. These results suggest that expression of 2-thiolation enzyme activity increases at higher temperatures. Thus, the 2-thiolation enzyme increases in both activity and abundance at higher temperatures, and these elevated levels could explain why the s^2T content of tRNA increases with the cultivation temperature.

Effect of Methylation of A58 for s^2T Biosynthesis—Analysis of the products of the 2-thiolation reaction in vitro suggested that methylation of A58 is required for s^2T biosynthesis (see above). To clarify the role of A58 methylation in 2-thiolation of rT54, we analyzed an m^1A-deficient strain of T. thermophilus HB8. Since trmI is known to encode the m^1A58 methyltransferase in T. thermophilus HB27 (13), we constructed a trmI knock-out strain of T. thermophilus HB8 and analyzed the modification of the tRNA (Fig. 5A). In this mutant, m^1A58 was completely lost, and the s^2T content decreased to 15% of that of the wild-type strain, as determined using a UV chromatogram (Fig. 5A). The mass spectrum of these modified nucleosides was also confirmed (data not shown). 2-thiouridine, which is known to be eluted near G, was not detected (data not shown). Thus, we conclude that in the trmI mutant, the thiolation reaction of rT54 to s^2T54 is severely inhibited.
FIGURE 5. m\textsuperscript{1}A dependence of s\textsuperscript{2}T biosynthesis. 
A, UV chromatograms of nucleoside analysis of the tRNA from HB8 (wild type) (a) and NS0802 (trmI::km) (b) using LC/MS. In the NS0802 strain, m\textsuperscript{1}A was completely missing, and the abundance of s\textsuperscript{2}T was ~15% of the wild-type level. m\textsuperscript{7}G, 7-methylguanosine; m\textsuperscript{1}G, 1-methylguanosine; m\textsuperscript{2}G, 2-methylguanosine; FA, 6-threonylcarnbamoyladenosine. B, thiolation analysis of tRNA\textsuperscript{Ile} of HB8 (lanes 1, 3, 5, and 7), and NS0802 (lanes 2, 4, 6, and 8), by APM PAGE. Intact tRNA\textsuperscript{Ile} (lanes 1, 2, 5, and 6) and the 3'-half fragment of tRNA\textsuperscript{Ile} (lanes 3, 4, 7, and 8) were analyzed in the normal gel (lanes 1–4) and the APM gel (85 µm APM) (lanes 5–8). Assignment of bands is shown on the right. C, time course of the in vitro thiolation reaction of the substrate tRNA with m\textsuperscript{1}A58 (squares) and without m\textsuperscript{1}A58 (filled circles). Each reaction was performed at 60°C. The assay was repeated in duplicate. AU, absorbance units.
We then analyzed the thiolation level of a single tRNA species using the APM-PAGE method. trnA<sup>Rho</sup> was 3′-labeled using [α-<sup>32</sup>P]dCTP, and in order to exclude the effect of s<sup>4</sup>U, the anticodon region was hydrolyzed by limited digestion with RNase T<sub>1</sub>. Intact tRNA<sup>Rho</sup> contains both s<sup>4</sup>U and s<sup>2</sup>T<sub>54</sub> (Fig. 5B, lanes 5 and 6). In the trmI strain, the s<sup>2</sup>T<sub>54</sub> content in the 3′-region of the tRNA<sup>Rho</sup> was about 20% of the wild type (Fig. 5B, lanes 7 and 8).

We further examined the effect of m<sup>1</sup>A<sub>58</sub> on 2-thiolation of tRNA <i>in vitro</i>. As substrate, we used the tRNA<sup>Met</sup> from the <i>E. coli</i> thiI mutant, with or without modification of m<sup>1</sup>A<sub>58</sub>. This was incorporated into tRNA by recombinant TrmI protein, and the modification was confirmed by RNA sequencing (data not shown). The lysate was prepared by centrifugation (100,000 × g) of the cell extract from the NS0801 (thiI::km), followed by gel filtration of the supernatant, in order to minimize methylation during incubation. In the <i>in vitro</i> assay system, tRNA with the m<sup>1</sup>A<sub>58</sub> modification was more effectively 2-thiolated than that without (Fig. 5C). These results suggest that 1-methylation of A58 is required for effective biosynthesis of s<sup>2</sup>T at 5 min of incubation, the difference in 2-thiolation between the modified and unmodified substrate tRNAs was about 2-fold, but after the longer incubation (>10 min), the extent of 2-thiolation of the m<sup>1</sup>A<sub>58</sub>-deficient substrate tRNA increased considerably when compared with the methylated tRNA. This is caused by the methylation of the m<sup>1</sup>A<sub>58</sub>-deficient tRNA by the lysate during the longer incubation period (confirmed by RNA sequencing; data not shown).

Finally, we examined the phenotype of the <i>trmI</i> mutant (NS0802), which has a particularly low s<sup>2</sup>T<sub>54</sub> content (about 15% of wild type; see Fig. 5A). This mutant exhibits a temperature-sensitive phenotype. Although the mutant and wild type demonstrate similar growth at 70 °C, the mutant can no longer grow at 80 °C, a temperature at which the wild type can still grow (Fig. 6A). Thus, this phenotype resembles the <i>trmI</i> strain of HB27 (13). Since the temperature sensitivity is thought to be caused by lowered thermostability of the tRNA, we compared the melting temperatures of total tRNA between the wild type (85.6 °C) and the <i>trmI</i> mutant (NS0802) (83.7 °C) and found a 2 °C difference (Fig. 6B). A58 methylation does not contribute to <i>T<sub>m</sub></i> (27); thus, it is evident that the lowered <i>T<sub>m</sub></i> of the total tRNA from the <i>trmI</i> mutant is the result of the decrease of the 2-thiouridylation (i.e. s<sup>2</sup>T is only 15% of the wild-type tRNA level). These results imply that s<sup>2</sup>T modification is responsible for both the thermal stability of the tRNA and the adaptation of <i>T. thermophilus</i> cells to higher temperatures. However, characterization of a mutant deficient in s<sup>2</sup>T only is necessary for a better understanding of the precise roles of s<sup>2</sup>T and m<sup>1</sup>A.

**DISCUSSION**

To date, there has been little reported on thermophile-specific tRNA s<sup>2</sup>T<sub>54</sub> biosynthesis, a process that confers thermal stability on tRNAs. In this paper, we characterized some aspects of the modification of rT to s<sup>2</sup>T, including the relevant enzymes and sulfur donors.

Considering the biosynthetic pathways for sulfur-containing modified nucleosides that have been recently characterized, it appears that activation of both the tRNA and the sulfur atom is necessary for the enzymatic reaction converting rT to s<sup>2</sup>T. It has been proposed that there are two distinct routes for the biosynthesis of thiolated nucleosides (e.g. s<sup>4</sup>U, mnm<sup>3</sup>s<sup>3</sup>U, ms<sup>3</sup>i<sup>4</sup>A, ms<sup>3</sup>io<sup>6</sup>A, and s<sup>3</sup>C) in tRNA (20, 21, 28, 29). First, the sulfur atom is transferred to a cysteine residue at the active site of IscS cysteine desulphurase (30, 31). In one pathway, sulfur is directly transferred from IscS to the tRNA-modifying enzyme in the persulfide form and then is finally transferred to the tRNA (32). In the other pathway, the sulfur on IscS is incorporated into the Fe-S clusters by the ISC machinery (IscSUA-HscBA-Fdx in <i>E. coli</i>) and is transferred to other protein(s) that have iron-sulfur clusters (e.g. the enzyme responsible for ms<sup>3</sup>i<sup>4</sup>A synthesis, MiaB, is known as an iron-sulfur protein (33–35)).

In this work, we first identified cysteine as one of the sulfur donors for biosynthesis of s<sup>2</sup>T. However, neither of the mutations <i>iscS</i> or <i>sufo</i> (a <i>iscS</i> parologue) affected the s<sup>2</sup>T content in <i>T. thermophilus</i>. In <i>E. coli</i> and
S. typhimurium, the sulfur atom for all thio-modifications of tRNAs is derived from cysteine, using the sulfur delivery protein IscS (20). Other cysteine desulfurases, SuF5 and CsdA (cysteine sulfinic dihydroxylase), are not involved in thio-modification (20). Strikingly, mutation of iscS did not alter s^2T biosynthesis, giving rise to speculation that there may be a supplementary role for SuF5 in s^2T synthesis in *T. thermophilus*, which could be investigated using a double mutant strain of iscS/suF5. However, in *E. coli*, the double deletion is lethal (24), and we suspect that a similar effect would occur in *T. thermophilus*, due to the lack of Fe-S cluster formation. An as yet unknown pathway that uses the sulfate ion may also exist in *T. thermophilus*, since the in vivo labeling experiment demonstrated that the sulfur atom from the sulfate ion is actually incorporated into the modified nucleosides of tRNA.

By constructing the in vitro assay system, we have successfully detected and characterized s^2T synthesis in vitro. The ATP requirement of the s^2T reaction was clearly observed in this system, and this is consistent with the fact that some tRNA modification enzymes are derived from P-loop ATPases (e.g. Thl (s^4U8 synthesis (32, 36)), MnmA (mmn^5s^2U34 (31)), Tis5 (lysidine 34 (37)), TtcA (s^2C32 (38)), and YbbB (s^2U34 (39)). It has been demonstrated that some of these enzymes bind the substrate tRNA (31, 32, 37, 38, 39)). However, it is likely that the sulfur transfer reaction we observed came from the S20 extract nor with the gel-filtrated S100 fraction (data not shown). Additionally, as seen with s^4U8 synthetase Thl, which has a rhodanese domain that serves as a sulfurtransferase (41, 42), a thiotransferase protein (or domain), together with the ATPase domain described above, may also be required for s^2T54 synthesis. In our in vitro assay, cysteine dependence was neither observed with the S20 extract nor with the gel-filtrated S100 fraction (data not shown). Thus, it is likely that the sulfur transfer reaction we observed came from an activated sulfur intermediate (possibly a persulfide or an Fe-S cluster) transferred by the modifying enzyme to the substrate tRNA.

Very little is known about the mechanisms controlling the amount of modified nucleosides, with the exception of the increase of methylation of G18 to 2’-O-methylguanosine 18 that has been observed in the tRNA of *T. thermophilus* HB27 in a high temperature environment. This increase may not necessarily be due to an increase in the abundance of enzyme but to an increase in methyltransferase activity (43). The s^2T content of tRNA in *T. thermophilus* HB8 increases with elevation of cultivation temperature in vivo (4). This phenomenon is an interesting strategy for bacterial adaptation to high temperature environments. Our in vitro results indicated that the molecular mechanism for this adaptation may be an increase in both the expression and activation of 2-thiolating enzyme(s) at higher temperatures. Since there may be various enzymes involved in this modification process (see above), these characteristics may be derived from properties of individual enzymes in this pathway. Identification of all of these enzymes and investigation of these properties will clarify the precise mechanism of the temperature dependence of s^2T biosynthesis.

Differences between the s^2T-thiolase from HB8 (this paper) and the Gm-methylase of HB27 (43) may indicate alternative mechanisms required for the control of the abundance of each post-transcriptional modification.

m^1A58 is required for effective 2-thiolation of rT54 both in vitro and in vivo (Figs. 3, 5, and 6), and this suggests that methylation of A58 is responsible for the recognition of the substrate tRNA in s^2T biosynthesis. In a recent paper (6), we identified the residues required for effective 2-thiolation of U54 and demonstrated that A58 shows a strict requirement for 2-thiolation. It is possible that 2-thiolation enzyme(s) recognize the positive charge resulting from methylation at the 1 position of A58. It is also possible that the TrmI protein may enable the 2-thiolase to recognize substrate tRNAs and that it is the TrmI protein itself, and not the methyl moiety of m^1A58, that is required for effective 2-thiolation of rT54. However, the addition of TrmI to the reaction mixture did not enhance the levels of 2-thiolation (data not shown).

During our investigation of the 2-thiolation of tRNA, characterization of the trmI mutant strain of *T. thermophilus* HB27 was published (13), and the temperature-sensitive phenotype of that strain was the same as for our HB8 mutant (NS8002) (Fig. 6). Droogmans’ group (13) proposes that m^1A58 is responsible for the thermal adaptation of *T. thermophilus*. However, aside from an m^1A deficiency, the trmI mutant strain has quite a low level of s^2T. We propose that the major reason for a lower tRNA Tm and for the temperature-sensitive phenotype is the reduction in the abundance of s^2T. Although the observed difference in the Tm of the tRNA between the wild-type and m^1A/s^2T-deficient strains is not great (about 2 °C; Fig. 6B), it may contribute significantly to the ability to grow in thermal environments over 80 °C. Characterization of a mutant that is deficient in s^2T only is necessary for clarification of the relative function of s^2T and m^1A residues in the thermal stabilization of tRNA.

Our future studies will involve the purification of the s^2T synthesis enzymes using the in vitro assay system in order to develop a clearer understanding of the mechanisms controlling the relative contents of rT and s^2T and their roles in temperature adaptation.

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