Identification of Two tRNA Thiolation Genes Required for Cell Growth at Extremely High Temperatures*

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Thermostability of tRNA in thermophilic bacteria is effected by post-transcriptional modifications, such as 2-thioribothymidine (s²T) at position 54. Using a proteomics approach, we identified two genes (ttuA and ttuB; RNA-two-thiouridine) that are essential for the synthesis of s²T in Thermus thermophilus. Mutation of either gene completely abolishes thio-modification of s²T, and these mutants exhibit a temperature-sensitive phenotype. These results suggest that bacterial growth at higher temperatures is achieved through the thermal stabilization of tRNA by a 2-thiolation modification. TtuA (TTCT0106) is a putative thio-carrier protein that exhibits significant sequence homology with ThiS of the thiamine synthesis pathway. Both TtuA and TtuB are required for in vitro s²T formation in the presence of cysteine and ATP. The addition of cysteine desulfurases such as IscS (TTCC0087) or SufS (TTC1373) enhances the sulfur transfer reaction in vitro.

EXPERIMENTAL PROCEDURES

Strains—The strains used for this study were wild-type T. thermophilus HB27 and T. thermophilus NS0801, which lacks the gene for 4-thio-uridine (s4U) biosynthesis (thiI) (5). Both wild-type and mutant strains were cultivated in rich medium (6) at 70°C without or with 30 µg/ml kanamycin, respectively, unless otherwise stated. Escherichia coli JM109 (7) and TOP10 (Invitrogen) were used as hosts for the genetic manipulation of plasmids.

Preparation of tRNA-bound Resin—The template for the in vitro transcription of tRNA<sup>le</sup> was constructed using PCR to amplify the tRNA sequence under control of the T7 promoter (8). The oligonucleotides used for the construction of a plasmid bearing the tRNA<sup>le</sup> gene are ile-temp-F (5'-ggcggagttttaaatgcagttctataggtgtaggccgacgcctgataaggctgag-3') and ile-temp-R (5'-tgatgccagttggacctgctgagctggctggcagtgggctgtgctga-3'). The PCR products were cloned into the EcoRI and HindIII sites of pUC19. For in vitro transcription, the pair of primers ile-F (5'-taaacagcaggtctataggtgtaggccgacgcctgataaggctgag-3')/ile-R (5'-tgatgccagttggacctgctgagctggctggcagtgggctgtgctga-3') was employed to PCR-amplify the template plasmid pUC19-tRNA<sup>le</sup>. Transcription of the tRNA gene was conducted 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 NTP, 5 mM GMP, 50 µg/ml bovine serum albumin, 2 µg/ml template DNA, and T7 RNA polymerase (8).

In order to prepare tRNA-bound resin, the 3'-ribose of the tRNA was oxidized to form a diahyde at room temperature, for 1 h in the dark, in a buffer containing 100 mM sodium acetate (pH 5.2), 10 mM sodium periodate, and 10 mM MgCl<sub>2</sub>. The oxidized tRNA was purified using a NAP 5 gel filtration column (Amersham Biosciences) and recovered by ethanol precipitation. Oxidized tRNA was biotinylated using Biotin (Long Arm) hydrazide (Vector), incubated in 100 mM sodium acetate (pH 5.2) and 10 mM MgCl<sub>2</sub>, at room temperature overnight in the dark. Unreacted biotin was removed by gel filtration using a NAP 5 column, followed by ethanol precipitation of the biotinylated tRNA. The precipi-
Identification of Two s² T Biosynthesis Genes

The pairs of oligonucleotides used were as follows: TTC0105-Nde-F (5'-ggcatGGATC Cttatagcca ggcccggtac ttggcctc-3') and TTC0105-Bam-R (5'-ggcatGATCc Tcagtcgag cgggggcttc-3'). The sequences complementary to the htk gene cassette were underlined. The PCR fragments containing the htk cassette insertion were cloned into pCR-BluntII-TOPO (Invitrogen) and sequenced.

Using the plasmids prepared as described above, T. thermophilus HB27 was transformed as previously described (11), and transformants were selected on rich medium containing 300 μg/ml kanamycin. Homologous recombination was confirmed using PCR amplification, followed by restriction digestion analysis.

Analysis of tRNA Modification—Cells were grown at 70 °C to late log phase, and total RNA was extracted using Isogen (Wako). The tRNA fraction (unbound fraction) was then applied to tRNA-Sepharose 4B, washed five times with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, and 150 mM KCl), and then bound to the resin with PCR-amplified TtuA and TtuB cassette (kanamycin resistance gene) was amplified from pUC18-pJHK3 (10). The supernatant that was again collected and subjected to desalting using a Nap 50 gel filtration column (Amersham Biosciences). About 30 mg of gel-filtrated S100 was obtained from a 1-liter culture of T. thermophilus HB27 (~1.4 g of cells).

In order to remove non-specific binding proteins, the desalted S100 fraction (2.5 mg) was mixed with Streptavidin-Sepharose HP and incubated for 10 min at room temperature, after which the resin was removed. The resultant fraction was then applied to tRNA-Sepharose (~50 μg of bound tRNA) and incubated at room temperature for 20 min, followed by washing three times with H buffer.

The resin-bound protein was mixed with an equal volume of sample buffer (125 mM Tris-HCl (pH 6.8), 2% SDS, 0.7 M 2-mercaptoethanol, and 0.02% bromphenol blue) and incubated at 95 °C for 5 min, prior to separation using SDS-PAGE. Proteins were visualized using Coomassie Brilliant Blue R-250, and bands of interest were excised and subjected to in-gel tryptic digestion as described previously (9). Tryptic digests were analyzed using nano-flow-HPLC-electrospray ionization tandem mass spectrometry. This consisted of a DiNa splitless nanoflow HPLC system (KYA Technologies) and a 50 × 0.15-mm inner diameter packed ODS (3-μm particle size) capillary column (KYA Technologies), which are used for efficient separation of small amounts of peptide. Tryptic digest fragments were separated in 0.1% formic acid in water, using a linear gradient from 0 to 100% of HCOOH for 40 min at a flow rate of 500 nl/min. Ionization of the eluted peptides was performed using LCQ ion trap mass spectrometry (Thermo Electron), through electrospray ionization (metal nanosprayer S; GL Sciences). Proteins were identified using the MASCOT data base search engine (Matrix Science) and compared with the T. thermophilus HB27 genome data base (NC_005835 and NC_005838 on NCBI).

Construction of ttuA::km and ttuB::km Strains—To construct the mutant strains ttuA::km (ttc0106::kn) and ttuB::km (ttc0105::kn) (NS2710 and NS2720, respectively), we PCR-amplified 0.5 kb from the 5'-flanking region of the mutant strains) at 70 °C overnight. Diluted culture (A600 = 0.1) and serial dilutions (10-1, 10-2, 10-3, and 10-4) were spotted onto rich medium plates and incubated for 31 h at 60 °C, 17 h at 70 °C, 15 h at 75 °C, 24 h at 80 °C, and 32 h at 82 °C.

Overexpression and Purification of TtuA, TtuB, IscS, and SufS Proteins—The gene encoding TtuA (TTC0106) of T. thermophilus HB27 was cloned into the Ndel and HindIII sites of PET22b (+) (Novagen), resulting in a C-terminal His₆-tagged fusion protein. The oligonucleotides used were TTC0106-Nde-F (5'-agccgGATCC taacgtgac gccggag-3') and TTC0106-Hin-R (5'-gagctAGATCT taccggag aggggga-3'). Proteins were identified using the MASCOT database search engine (Matrix Science) and compared with the T. thermophilus HB27 genome data base (NC_005835 and NC_005838 on NCBI).
Identification of Two s^2T Biosynthesis Genes

tein TtuA and the co-expressed TtuA/TtuB were purified as above, except for the addition of 500 mM KCl to all buffers and 200 mM imidazole to the buffer for gel filtration of TtuA in order to prevent precipitation. Centricon columns YM10 and YM3 were used for concentration of TtuA and TtuA/TtuB, respectively. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin standard. Glycerol was added to the purified protein solutions to a final concentration of 30%, and each sample was stored at −30 °C.

2-Thiolation Assay with Recombinant Proteins—tRNAPhe from Saccharomyces cerevisiae (Sigma) was dephosphorylated using bacterial alkaline phosphatase (Takara), and T4 polynucleotide kinase (Toyobo) was used to label the 5′ terminus with [γ-32P]ATP (110 TBq mmol−1; Amersham Biosciences), followed by purification with denaturing 10% PAGE.

The 2-thiolation assays were performed at 60 °C for 20 min in 20 μl of H buffer containing 5 mM ATP, 1 mM cysteine, 20 μM pyridoxal 5′-phosphate, 5′-labeled tRNAPhe (−50,000 cpm/−10 ng), 100 μg of desalted S100 (from the NS0801 strain that had been grown at 80 °C), and 30 pmol of the recombinant proteins. tRNA was recovered using Isogen (Wako), ethanol-precipitated, and separated using [(N-acrylamino)phenyl]mercuric chloride (APM)-containing PAGE (100 μM APM) (13, 14). Gels were exposed to an imaging plate, followed by analysis using a BAS1000 bioimaging analyzer (Fuji Photo Systems).

Identification of Two s^2T Biosynthesis Genes

The 2-thiolation reaction with [35S]cysteine was performed as described above with the following modifications. Four μg of S. cerevisiae tRNAPhe and 20 μM [35S]cysteine (20 μCi) were added to 20 μl of reaction mixture. [35S]cysteine was purchased from American Radiolabeled Chemicals. RNAs were recovered using Isogen reagent and ethanol-precipitated. For alkaline treatment, the RNA sample was incubated at 37 °C for 1 h in 100 mM HEPEs-KOH (pH 9.0). The sample was then subjected to 10% PAGE using gels containing 7 M urea, and the gels were then stained with EtBr. The gel was dried, exposed to an imaging plate, and analyzed using a BAS 1000 bioimaging analyzer (Fuji Photo Systems). For nucleoside analysis, labeled tRNAPhe was purified by PAGE, and 35S-labeled nucleosides were analyzed by an HPLC system as essentially described in the literature (5). Fractions, which were collected every 37.8 s, were measured for absorbance at 280 nm and radioactivity.

RESULTS

Purification of tRNA-binding Proteins from T. thermophilus Cell Extract—In order to identify the gene encoding the protein responsible for tRNA thiolation, we isolated the tRNA-binding proteins from a cell extract of T. thermophilus and identified the corresponding genes using proteomics. Transcript tRNAPhe was biotinylated with ~90% efficiency (data not shown) and immobilized on a streptavidin-Sepharose resin. The immobilized tRNAPhe was used for the affinity purification of tRNA-binding proteins from a cell extract of T. thermophilus (Fig. 1). Samples were separated using a gradient SDS-PAGE. When compared, the sample that was affinity-purified with tRNA-Sepharose (lane 3) exhibited a different pattern from that of the cell extract prior to purification (lane 1), and little nonspecific binding to the resin alone was observed (lane 2). This suggests that tRNA-binding proteins were purified successfully.

The major protein bands in lane 3 were excised and analyzed using mass spectrometry. A total of 108 proteins were identified from 2,204 ORFs of T. thermophilus HB87. Of these, ~40% were predicted to interact with RNA (tRNA, rRNA, and mRNA); the remainder comprised DNA-binding proteins, biotin-binding proteins, metabolic pathway proteins, and proteins of unknown function.

Among the RNA-binding proteins identified, we found 14 tRNA-binding proteins; tRNA nucleotidyltransferase (CCA-adding enzyme), elongation factor Tu, Ile-tRNA-synthetase, peptidyl-tRNA hydrolase, and 10 tRNA-modifying enzymes (Fig. 1B). We used the tRNA^ile species for the purification experiment, since native tRNA^ile has eight post-transcriptional modifications (15): 4-thiouridine at position 8 (s^4U8); 2′-O-methylguanosine at position 18 (Gm18); dihydrouridine at position 20a (D20a); 6-threonylcarbamoyladenosine at position 37 (C^6A37); 7-methylguanosine at position 46 (m7G46); s^2T54; pseudouridine at position 55 (Ψ55); and 1-methyladenosine at position 58 (mA^1A58). With the exception of the enzymes for 2-thiolation of s^2T and 6-threonylation of t^A, all of the other enzymes responsible for the modification of tRNA^ile have been identified (i.e. Thil for s^4U8 (16), TrmH for Gm18 (17), Dus for D20a (18), TrmB for mA^1A58 (19), TrmFO for t^T54 (20), TruB for Ψ55 (21), and TrmI for mA^1A58 (22)). Additionally, enzymes that are not involved in modification of tRNA^ile were identified, such as TruD for Ψ13 (23), MiaA for 6-isopentenyladenosine at position 37 (C^6A37) (24), and TruA for Ψ38–40 (25). Considering these results, enrichment of modification enzymes from the cell extract turned out to have been very efficiently achieved by our strategy.

Identification of the Genes Essential for the 2-Thiouridylation Reaction—Among the proteins in the affinity-purified sample, we identified one belonging to the TtcA family (TTC0106) (Figs. 1A, 2A, and 3A). This pro-
tein family includes TtcA, an enzyme that is responsible for the biosynthesis of 2-thiocytidine (s²C) at position 32 of tRNA (26). The amino acid sequence of TTC0106 indicates that it is more closely related to the Group II TtcA family of proteins. These proteins possess a P-loop motif (Ser-Gly-Gly-Xaa-Asp-(Ser/Thr)) and four or five Cys-Xaa-Xaa-Cys (CX2C) motifs. In contrast, TtcA from Escherichia coli is included in Group I, which possesses a P-loop motif and two CX2C motifs. The P-loop motif specifically binds ATP to form an adenylated intermediate; other tRNA-modifying enzymes, such as MnmA, Thil, and Tis, also possess this motif (27–29). By the analysis of tRNA modification using liquid chromatography/mass spectrometry (LC/MS), we were unable to detect s²C in the tRNA mixture of T. thermophilus under similar conditions to those required for detection of s²C from E. coli (data not shown). Thus, we believe that TTC0106 is not involved in s²C biosynthesis but is a candidate for s²T biosynthesis.

TTC0105 was also affinity-purified using tRNA-Sepharose (Figs. 1A and 2B). It is a paralog of ThiS, a sulfur carrier protein essential for biosynthesis of the sulfur-containing cofactor, thiamine (30, 31). Multiple alignment of TTC0105 and related sequences indicates that these proteins possess a conserved C-terminal Gly-Gly motif (Fig. 3B). The C terminus of ThiS is thio-carboxylated with a sulfur atom from cysteine, and then the sulfur atom is incorporated into the thiamine precursor (31, 32). TTC0316 appears to be an ortholog of ThiS, and not only does the sequence closely resemble ThiS from E. coli (Fig. 3B), but in the T. thermophilus genome, it is located in a thiamine biosynthesis operon (ThiESGOC, unknown ORF, and ThiD; TTC0315-0321).

The genes encoding TTC0105 and TTC0106 form a single operon (Fig. 4A) and are probably transcribed from a transcriptional start site immediately upstream of the TTC0105 coding region. To examine whether or not TTC0105 and TTC0106 are involved in s²T biosynthesis, we constructed insertion mutants of ttc0106 or ttc0105 and analyzed the modified nucleosides of unfractionated tRNA using LC/MS. In order to avoid polar effects in the ttc0105 insertion strain, ttc0106 was constructed so as to be transcribed together with a drug resistance gene.
In both mutants, s^2T (27.7 min) was completely absent, although its precursor r^T (21.4 min) was detected (Fig. 4B); other nucleosidemodifications remained unchanged. These results suggest that both TTC0105 and TTC0106 are involved in s^2T biosynthesis. A small peak (27.5 min) from dinucleotide GmpG was observed immediately before the s^2T peak and was derived from incomplete digestion of tRNA. This assignment was confirmed by the mass spectrum (m/z = 643) and by the disappearance of the peak in trmH, a Gm18-methylase mutant (data not shown). Thus, we have renamed the proteins TTC0106 and TTC0105 to TtRNAs^2-thiouridine-synthesizing protein A and B (TtuA and TtuB), respectively, and the corresponding genes are now designated tttuA and tttuB. The precursor of s^2T was identified as r^T, suggesting that the 5-methyltransferase that modifies U at position 54 (TrmFO) (20) does not recognize the 2-thiocarbonyl group of s^2U.

**Temperature-sensitive Phenotypes of s^2T-deficient Strains**—Although the 2-thiocarbonyl group of s^2T^54 is known to be responsible for the thermostabilization of tRNA (1, 3) and tRNA thus modified functions effectively at elevated cultivation temperatures (3), a direct requirement for the thermal stabilization of tRNA in high temperature environments has not been demonstrated.

The growth phenotypes of two s^2T-deficient strains, NS2710 (ttuA::km) and NS2720 (ttuB::km), were examined. These insertion
mutants were cultivated at various temperatures on rich medium plates. Although neither ttu mutant exhibited any obvious growth defect when cultivated below 75 °C, both mutants were unable to grow above 80 °C (Fig. 5). This is the first direct evidence that thermal stabilization of tRNA, accomplished by the s^2T modification, is required for cell growth at high temperatures.
In Vitro Formation of s^{2}T Using Recombinant TtuA and TtuB—We cloned the genes encoding TtuA and TtuB into His-tagged expression vectors and overexpressed the proteins in E. coli. When TtuA and N-terminal hexahistidine (His6)-tagged TtuB were co-expressed in E. coli, the untagged TtuA was co-purified with the His6-tagged TtuB during Ni^{2+}/HCl affinity column chromatography (Fig. 6A, lane 1). This suggests that these two proteins are able to form a heterocomplex. However, the ratio of the two proteins varied in each preparation (data not shown), indicating that the complex is not stable under the conditions used in this study. Therefore, we used individually expressed proteins for the experiments. TtuA and TtuB were expressed and purified to ~90% homogeneity (Fig. 6A, lanes 2 and 3). Both proteins were colorless, and the UV-visible adsorption spectra did not indicate the presence of an iron sulfur (Fe-S) cluster (data not shown).

In vitro s^{2}T formation was assayed with tRNA^{Phe} from Saccharomyces cerevisiae using thiocarbonyl-affinity electrophoresis (5). The extent of thiolation was determined by retardation of the thiolated tRNA (13, 14). Initially, we attempted to reconstitute the thiolation reaction using only the TtuA and TtuB proteins, but no thiolation was observed (data not shown). We then performed the reaction in the presence of the gel-filtered cell extract (S100) from the s^{4}U-deficient mutant (NS0801, thiI mutant strain of T. thermophilus (5)) in order to avoid the formation of s^{4}U. As shown in Fig. 6B, lane 1, a small amount of upper-shifted band was observed that may have been 2-thiolated by S100 only. Although neither the addition of TtuA nor TtuB significantly increased 2-thiolation levels (Fig. 6B, lanes 1–3), there was significant enhancement when both were added to the reaction mixture (lane 4). Although this result clearly indicates that both TtuA and TtuB are involved in the biosynthesis of s^{2}T, other component(s) present in the S100 extract are also required for the reaction.

Since TtuA and TtuB are predicted to be an ATPase and a sulfur carrier, respectively (see above), ATP and cysteine were omitted from the reaction mixture.
Identification of Two s\textsuperscript{2}T Biosynthesis Genes

FIGURE 7. Incorporation of [\textsuperscript{35}S] sulfur into s\textsuperscript{2}T in vitro. A, PAGE analysis of [\textsuperscript{35}S] sulfur incorporation into tRNA. The in vitro reacted RNAs were first visualized by EtBr staining (upper panel), and then [\textsuperscript{35}S] radioactivities were visualized on the imaging plate (IP, lower panel). The in vitro reaction was carried out with S100 alone (lanes 1–4); S100, TuuA, Tuub, and IscS (lanes 5–8); or S100, TuuA, Tuub, and SufS (lanes 9–12). S. cerevisiae tRNA\textsuperscript{Phe} was added to lanes 3, 4, 7, 8, 11, and 12. After the reaction, the samples were treated with alkaline (lanes 2, 4, 6, 8, 10, and 12). The positions of S. cerevisiae tRNA\textsuperscript{Phe} (S. c. tRNA\textsuperscript{Phe}), S. cerevisiae tRNA\textsuperscript{TM} (S. c. tRNA\textsuperscript{TM}), tRNA mixture from S100 (tRNAs), and tRNA\textsuperscript{CM} from S100 (T. t. tRNA\textsuperscript{CM}) are indicated on the right B. The nucleoside analysis of [\textsuperscript{35}S] labeled tRNA\textsuperscript{TM} (reacted with S100, TuuA, Tuub, and IscS; from lane 8 of A) by reverse phase chromatography. The squares indicate UV absorvance at 280 nm. The circles represent \textsuperscript{35}S radioactivity (cpm). The elution position of co-injected authentic s\textsuperscript{2}T is indicated by an arrow.

The reaction mixture (Fig. 6C). Elimination of ATP resulted in a complete loss of s\textsuperscript{2}T synthesis (lane 2), whereas elimination of cysteine merely resulted in a moderate decrease in the reaction (lane 3), and it is possible that a sulfur intermediate from the cell extract was used for the residual s\textsuperscript{2}T synthesis (5). These results demonstrate that s\textsuperscript{2}T synthesis requires both ATP and cysteine. When ATP was removed from the reaction mixture, the substrate tRNA was slightly degraded (shown by an asterisk in Fig. 6C). ATP may protect tRNA from degradation by an unknown mechanism.

IscS and SufS Enhance s\textsuperscript{2}T Formation—IscS (iron sulfur cluster) is a cysteine desulfurase that is responsible for biosynthesis of thio-modifications in E. coli (33) and Salmonella typhimurium (34). This enzyme catalyzes the initial sulfur transfer reaction from cysteine. Also in the case of T. thermophilus, it is supposed that the sulfur atom of cysteine is finally incorporated into s\textsuperscript{2}T in vivo (5). Thus, IscS is one of the candidate enzymes for s\textsuperscript{2}T biosynthesis.

Sequences encoding two cysteine desulfurases, IscS and SufS, were identified from the T. thermophilus genome. These genes were cloned, overexpressed as N-terminal His\textsubscript{6}-tagged IscS (TTTC0087) and SufS (TTTC1373) fusion proteins in E. coli, and purified to \textasciitilde90% homogeneity (Fig. 6A, lanes 4 and 5). These proteins were yellow, a feature characteristic of pyridoxal 5'-phosphate-binding proteins (35), and their UV-visible spectrum indicated the presence of pyridoxal 5'-phosphate (data not shown). Although neither the addition of IscS nor SufS significantly increased 2-thiolation levels (Fig. 6B, lanes 7 and 8), both cysteine desulfurases increased 2-thiolation in vitro in the presence of TuuA/B (Fig. 6B, lanes 5 and 6); \textasciitilde40% of the substrate tRNA became 2-thiolated in the presence of SufS (lane 6). These results suggest the involvement of these cysteine desulfurases in s\textsuperscript{2}T synthesis.

The Sulfur Atom of [\textsuperscript{35}S] Cysteine Is Incorporated into s\textsuperscript{2}T in Vitro—Next, we performed in vitro tRNA thiolation reactions with [\textsuperscript{35}S]cysteine and unlabeled S. cerevisiae tRNA\textsuperscript{Phe}. After the reaction, the RNAs were separated by denaturing PAGE, and the incorporation of [\textsuperscript{35}S] sulfur into the RNAs was monitored by applying an imaging plate (Fig. 7A). When no recombinant proteins were added, faint bands corresponding to 5 S rRNA and the tRNA mixture derived from T. thermophilus S100 (lane 1) were detected in the EtBr-stained gel. tRNA\textsuperscript{Phe} was clearly visible by EtBr staining when added to the reactions (lane 3). One major \textsuperscript{35}S-labeled species was detected (lanes 1 and 3) by measuring the radioactivity in the gel, and the bands disappeared after mild alkaline treatment of the samples (lanes 2 and 4). Thus, these bands could be derived from \textsuperscript{35}S[cysteinylated tRNA\textsuperscript{Cys}] species that were originally present in the T. thermophilus S100 fraction and that were cysteinylated during incubation.

When recombinant TtuA and TtuB and IscS or SufS were added (lanes 5–8 or 9–12, respectively), substrate tRNA\textsuperscript{Phe} was \textsuperscript{35}S-labeled together with tRNA\textsuperscript{CM} from the S100 fraction (lanes 7 and 11). The \textsuperscript{35}S labels of tRNA\textsuperscript{Phe} were tolerant of mild alkaline treatment (lanes 8 and 12), suggesting that these bands are derived from tRNA\textsuperscript{Phe} modified by \textsuperscript{35}S.

In order to confirm that the sulfur atom of cysteine was actually incorporated into s\textsuperscript{2}T, we analyzed the modifications of these labeled tRNA\textsuperscript{Phe} (lanes 8 and 12). Labeled tRNA\textsuperscript{Phe} was first purified by PAGE and then digested into nucleosides before being analyzed by ODS reversed phase column chromatography. One major \textsuperscript{35}S peak (31.2 min) was detected in the tRNA hydrolysate (Fig. 7B). The elution position of this peak was the same as that of authentic s\textsuperscript{2}T that was co-injected with the sample (shown by an arrow). The chromatogram pattern was essentially the same for tRNAs reacted with IscS (Fig. 7A, lane 8) and SufS.
Identification of Two $s^2T$ Biosynthesis Genes

In order to identify tRNA modification enzymes, we used immobilized tRNA$^{14}$ to affinity-purify proteins that bound to tRNA. All of the known tRNA-metabolizing enzymes were purified from the T. thermophilus cell extract. Therefore, this approach proved to be an effective method for the identification of RNA-modifying enzymes and other proteins that bind RNA.

We identified two candidate genes and identified their roles in $s^2T$ biosynthesis using insertional mutagenesis. Given what is known about the tRNA modification mechanism and sulfur transfer reaction in thiamine biosynthesis, we suspect that TtuA may interact with and activate tRNA and that TtuB may function as an intermediate sulfur donor. The in vitro assay indicated that the cysteine desulfurases, IscS and SufS, were also involved in $s^2T$ biosynthesis. A putative $s^2T$ biosynthesis pathway is presented in Fig. 8, and the potential roles played by TtuA, TtuB, IscS, and SufS are discussed below. Although the first activation of the sulfur atom from cysteine is achieved by the cysteine desulfurase in all of the known thio-modification pathways (33, 34), the involvement of TtuB-like sulfur carrier protein is a novel feature specific for the $s^2T$ biosynthesis pathway.

TtuA contains a P-loop motif (Ser-Gly-Gly-Xaa-Asp-(Ser/Thr)), which specifically binds ATP to form an adenylate intermediate (36–38). This motif is found on tRNA-binding ATPases, such as MmmA, ThiI, and ThiS (27–29), which activate target nucleosides by forming an adenylate intermediate (39). We propose that TtuA may be a tRNA-binding (activating) protein, since (a) it possesses a P-loop motif, (b) the in vitro 2-thiolation reaction has an ATP requirement, and (c) the protein was identified initially in a tRNA binding experiment (Fig. 1).

TtuA has a number of Cys-Xaa-Xaa-Cys (CXXC) motifs, which are found at the redox centers of thioredoxins, and disulfide bond isomerase (40, 41). This motif is also known to act as a metal binding site, either for divalent cation such as Zn$^{2+}$ (42), or for iron sulfur (Fe-S) clusters (43). In this study, no evidence was found for an Fe-S cluster in the UV-visible absorption spectrum of the recombinant TtuA. However, many Fe-S clusters are known to be unstable under oxidative conditions, and further experimentation is required to confirm the absence of an Fe-S cluster in TtuA. TtcA, a protein I member of the TtuA/TtcA family, may have an Fe-S cluster, because the synthesis of $s^2C$ requires IscU (44), which is involved in Fe-S cluster biogenesis.

The presence and absence of Fe-S clusters may be specific features of groups I and II members of the TtuA/TtcA family, respectively. The cysteines in the central CXXC motif of TtcA are known to be essential for $s^2C$ formation in S. typhimurium (26) and may play a similar role in TtuA for the synthesis of $s^2T$.

TtuB is a close paralog of ThiS, a sulfur carrier protein of the thiamine biosynthesis pathway (32). Thiamine is a sulfur-containing cofactor essential for enzymes involved in carbohydrate and branched-chain amino acid metabolism and is synthesized from thiazole and pyrimidine moieties (32). A sulfur atom that is derived from cysteine is eventually incorporated into the thiazole. The sulfur transfer pathway, from cysteine to thiazole, uses a C-terminal thiocarboxylate of ThiS (Thi$S$-COSH) as an intermediate sulfur donor. The thiocarboxylate is formed in two stages. Another ATPase, ThiF, acyl-adenylates the C-terminal of ThiS to ThiS-COAMP (30). ThiS-COAMP is then converted to the thiocarboxylate form (Thi$S$-COSH) by ThiF and IscS, using cysteine as a sulfur donor (31). Similar activation pathways are found in the biosynthesis of molybdenum cofactor (45) and in the eukaryotic ubiquitin conjugation system (46).

Given that TtuB shares significant sequence homology with ThiS (Fig. 38), it also may be thiocarboxylated at the C-terminal glycine (TtuB-COSH) by IscS or SufS using cysteine as a sulfur donor, and this putative thiocarboxylate may be the immediate sulfur donor for $s^2T$ formation. In this scenario, the sulfur atom of TtuB-COSH would be incorporated into $s^2T$, either by direct transfer to the tRNA or via TtuA (possibly to a conserved cysteine residue). If this scenario is correct, then we would expect a C-terminal acyl-adenylated form of TtuB (TtuB-COAMP) to be synthesized by an ATPase (corresponding to the activity of ThiF in ThiS-COAMP synthesis). The results of our in vitro $s^2T$ reaction demonstrated that ATP was an absolute requirement for $s^2T$ synthesis (Fig. 6), suggesting the involvement of a TtuB-activating ATPase, in addition to the tRNA-activating ATPase (TtuA). Although it is possible that TtuA is responsible for the C-terminal activation of TtuB, a TtuB-activating ATPase is a major candidate for one of the unidentified but strictly required components of the in vitro $s^2T$ reaction, present in the T. thermophilus cell extract. Both IscS and SufS enhanced formation of $s^2T$ in the in vitro reaction (Fig. 6). This result also strongly suggests the involvement of a C-terminal thiocarboxylated form of TtuB (TtuB-COSH). In Bacillus subtilis, all four cysteine desulfurases (CSD, NifZ, YrvO, and NifS) can serve as sulfur donors for the formation of Thi$S$-COSH (47). IscS is a primary sulfur donor for the thio-modifications of tRNA in E. coli (33). However, in T. thermophilus, mutants of IscS and SufS remain capable of synthesizing $s^2T$
Identification of Two $s^2T$ Biosynthesis Genes

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Identification of Two s²T Biosynthesis Genes

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