Common thiolation mechanism in the biosynthesis of tRNA thiouridine and sulphur-containing cofactors

Naoki Shigi^{1,*}, Yuriko Sakaguchi²,
Shin-ichi Asai³, Tsutomu Suzuki²
and Kimitsuna Watanabe^{1,*}

¹Biomedical Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan,

²Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Tokyo, Japan and ³Japan Biological Information Research Center (JBIRC), Japan Biological Informatics Consortium (JBIC), Tokyo, Japan

2-Thioribothymidine (s^2T), a modified uridine, is found at position 54 in transfer RNAs (tRNAs) from several thermophiles; s^2T stabilizes the L-shaped structure of tRNA and is essential for growth at higher temperatures. Here, we identified an ATPase (tRNA-two-thiouridine C, TtuC) required for the 2-thiolation of s^2T in *Thermus thermophilus* and examined *in vitro* s^2T formation by TtuC and previously identified s^2T -biosynthetic proteins (TtuA, TtuB, and cysteine desulphurases). The C-terminal glycine of TtuB is first activated as an acyl-adenylate by TtuC and then thiocarboxylated by cysteine desulphurases. The sulphur atom of thiocarboxylated TtuB is transferred to tRNA by TtuA. In a *ttuC* mutant of *T. thermophilus*, not only s^2T , but also molybdenum cofactor and thiamin were not synthesized, suggesting that TtuC is shared among these biosynthetic pathways. Furthermore, we found that a TtuB–TtuC thioester was formed *in vitro*, which was similar to the ubiquitin-E1 thioester, a key intermediate in the ubiquitin system. The results are discussed in relation to the mechanism and evolution of the eukaryotic ubiquitin system.

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Introduction

One of the characteristic features of RNA molecules is their post-transcriptional modifications. Among functional RNA

species, transfer RNA (tRNA) is extensively modified during its maturation steps, and these modifications are required for the tRNA to fulfil its functions in translation: codon recognition, maintenance of reading-frame, stabilization of tertiary structure, and serving as identity elements for the translation machinery (Curran, 1998).

In thermophilic organisms such as *Thermus thermophilus* and *Pyrococcus furiosus*, tRNA is stabilized by a specific thiomodification. In these organisms, the ribothymidine (rT) at position 54 is further modified to 2-thioribothymidine (s^2T) (Figure 1A) (Watanabe *et al*, 1974; Kowalak *et al*, 1994). The 2-thiolation level of rT54 in tRNA increases with culture temperature of the bacteria, and the melting temperature of the tRNA increases concomitantly with s^2T incorporation (Watanabe *et al*, 1976; Kowalak *et al*, 1994). In addition, 2-thiolated tRNA functions more efficiently at higher temperatures in an *in vitro* translation system (Yokoyama *et al*, 1987). These findings suggest that by controlling the 2-thiolation level of rT54, the protein synthesis machinery is optimized for variations in environmental temperature, and we have recently reported that 2-thiolation of tRNA is indispensable for growth at high temperatures (Shigi *et al*, 2006b).

We have previously identified four proteins required for the thiolation of rT54 in *T. thermophilus* tRNA: two cysteine desulphurases (IscS or SufS) and two tRNA-two-thiouridine synthesizing proteins (TtuA and TtuB) (Shigi *et al*, 2006a,b) (Figure 1A). Using these proteins and a *T. thermophilus* cell extract, *in vitro* formation of s^2T could be reconstituted in the presence of cysteine and ATP. These data suggest that there must exist other unidentified factor(s) required for s^2T biosynthesis. Additionally, s^2T biosynthesis may share similar chemistry with molybdenum cofactor (Moco) and thiamin synthesis, as described below.

Moco and thiamin are sulphur-containing cofactors (Figure 1B) whose biosynthesis includes a key sulphur transfer step that uses unique sulphur carrier proteins. Moco is incorporated into the active sites of many molybdoenzymes, including nitrate reductase, sulphite oxidase, and xanthine dehydrogenase (Schindelin *et al*, 2001). The Moco biosynthetic pathway is conserved across all domains of life. Moco contains a molybdenum atom and a pterin named molybdopterin (MPT) (Figure 1B). In MPT biosynthesis, two sulphur atoms are incorporated into a precursor (precursor Z) using a protein thiocarboxylate as a sulphur donor. In *Escherichia coli*, MoaD is used as a sulphur carrier (Figure 1Ca). An ATPase, MoeB, acyl-adenylates the C-terminal glycine of MoaD to form MoaD-COAMP (Leimkühler *et al*, 2001), which is then converted to the thiocarboxylate form (MoaD-COSH) by cysteine desulphurases (Leimkühler and Rajagopalan, 2001). Finally, sulphur atoms from two MoaD-COSH are transferred to precursor Z to form MPT (Pitterle and Rajagopalan, 1993).

*Corresponding authors. N Shigi, Biomedical Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan. Tel.: +81 3 3599 8265; Fax: +81 3 3599 8579; E-mail: naoki-shigi@aist.go.jp or K Watanabe, Biomedical Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan. Tel.: +81 3 3599 8106; Fax: +81 3 5530 2064; E-mail: kim-watanabe@aist.go.jp

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Figure 1 Biosynthesis pathways for s²T and other sulphur-containing cofactors. **(A)** Biosynthesis of 2-thioribothymidine (s²T) from ribothymidine (rT). We identified a new biosynthetic gene, TtuC. The requirement for other proteins or cofactors remains to be investigated. **(B)** Chemical structures of molybdenum cofactor (Moco) and thiamin. The molybdopterin (MPT) of Moco and the hydroxyethylthiazole (HET) moiety of thiamin are boxed by dotted lines. **(C)** Comparison of the biosynthesis pathways of sulphur-containing molecules (a–c) and the eukaryotic ubiquitin (Ub) conjugation system (d). In Moco synthesis (a), a thiocarboxylate (MoaD-COSH) serves as sulphur donor. Although cysteine desulphurases provide the sulphur atom for MoaD-COSH in *E. coli*, the *in vivo* sulphur source for MoaD-COSH has not been clarified in humans. In thiamin synthesis (b), a thiocarboxylate (ThiS-COSH) serves as the sulphur donor. An acyl-disulphide complex of ThiS–ThiF was detected in the *E. coli* pathway. In s²T synthesis (c), we analysed three steps and the intermediates in this study. In the Ub system (d), Ub–E1 thioester is formed as an important intermediate. The characteristic intermediates are boxed.

Thiamin is an essential cofactor for enzymes involved in carbohydrate and branched-chain amino acid metabolism and is synthesized from thiazole (hydroxyethylthiazole; HET) and pyrimidine (hydroxymethylpyrimidine) moieties (Figure 1B) (Settembre *et al*, 2003). The sulphur atom of the thiazole ring of HET is added in most bacteria by a system similar to the Moco biosynthetic machinery (Figure 1Cb): ThiS (a sulphur-carrier protein), ThiF (an ATPase), and IscS (a cysteine desulphurase) (Taylor *et al*, 1998; Lauhon and Kambampati, 2000).

By comparing the genes required for the formation of s²T in tRNA with those for the formation of Moco and thiamin, the function of each protein in s²T biosynthesis is predicted as follows (Figure 1Cc). Considering the sequence similarity with MoaD/ThiS (Supplementary Figure S1A), TtuB is a putative sulphur-carrier protein and may be first activated by an unidentified ATPase (ThiF/MoeB homologue) to form an acyl-adenylate, which is then thiocarboxylated by a cysteine desulphurase. Another putative ATPase, TtuA, may

activate the target nucleoside of tRNA and transfer the sulphur atom from the TtuB thiocarboxylate to form s²T in tRNA.

Furthermore, there is some limited similarity between the biosynthetic pathways of these sulphur-containing molecules and the eukaryotic ubiquitin system. Ubiquitin (Ub) and the ubiquitin-like modifier (Ubl) are protein modifiers involved in protein degradation and regulation of diverse cellular processes in eukaryotes, and the abnormalities in the Ub/Ubl system often causes serious diseases in humans (Hershko and Ciechanover, 1998; Kerscher *et al*, 2006). In the first step of Ub/Ubl conjugation, the C-terminal glycine is acyl-adenylated by an activating enzyme (E1) and covalently linked to a cysteine residue of E1 to form a Ub/Ubl–E1 thioester intermediate (Figure 1Cd). The activated Ub/Ubl is then transferred to a conjugating enzyme (E2). Finally, Ub/Ubl is attached to a lysine residue in the target protein by a ligase (E3). The first adenylation step is common to the sulphur cofactor biosynthesis pathways and the Ub/Ubl

system, implying an evolutionary link between these systems (Hochstrasser, 2000).

Here, we identified a new gene (*ttuC* (*two-thiouridine C*), a *thiF/moeB* homologue) responsible for the synthesis of s²T in *T. thermophilus*, and demonstrated that thiocarboxylated TtuB functions as sulphur donor for s²T synthesis *in vitro*. We found that *ttuC* was responsible for the synthesis of Moco and thiamin. Furthermore, we found that a TtuB–TtuC thioester can be formed *in vitro*. This is the first observation of a protein thioester similar to Ub/Ubl-E1 in a prokaryotic system, and the evolutionary implications for the origin of the eukaryotic protein-modification system are discussed.

Results

Identification of a novel gene required for s²T synthesis

We searched for MoeB/ThiF homologues, which may activate the putative sulphur carrier, TtuB. Surprisingly, a Blast search with the *E. coli* MoeB and ThiF found only one homologue (TTC0979) in the *T. thermophilus* HB27 genome, although independent biosynthetic pathways for Moco and thiamin may exist. TTC0979 has a P-loop and two Cys-X-X-Cys motifs (Supplementary Figure S1B, Figure 2A). This protein family is conserved across all domains of life (Krepinsky and Leimkühler, 2007). The P-loop motif of MoeB (Leimkühler *et al*, 2001) and ThiF (Duda *et al*, 2005) functions as an ATPase. The two CXXC motifs bind a Zn²⁺ ion, which stabilizes the structure of the protein (Lake *et al*, 2001; Duda *et al*, 2005).

To investigate whether TTC0979 is involved in s²T biosynthesis, we constructed a deletion mutant of *ttc0979* (NS2730) from *T. thermophilus* HB27 and analysed the modified nucleosides of unfractionated tRNAs by liquid chromatography/mass spectrometry (LC/MS). s²T (retention time: 28.4 min) was detected in wild-type tRNAs, but not in NS2730 tRNAs (Figure 2B). In NS2730, the precursor rT was detected (21.8 min) and other nucleoside modifications remained unchanged. This result clearly indicates that *ttc0979* is involved in s²T biosynthesis. Thus, we have renamed the *ttc0979* gene tRNA two-thiouridine-synthesizing protein C (*ttuC*). The NS2730 (*ttuC::km*) strain exhibited growth defects at temperatures higher than 80°C (Figure 2C), similar to the *ttuA* and *ttuB* mutants (Shigi *et al*, 2006b).

TtuB-COSH is formed by cysteine desulphurases and TtuC

Considering the weak but overall sequence similarity of TtuB to the sulphur carrier in Moco and thiamin biosynthesis (Supplementary Figure S1A), TtuB is predicted to be thiocarboxylated and to serve as sulphur donor in s²T synthesis. Therefore, we examined TtuB-COSH formation *in vitro* using recombinant proteins. Incorporation of ³⁵S-sulphur into these proteins from ³⁵S-labelled cysteine was analysed by non-reducing SDS-PAGE (Figure 3). The cysteine desulphurases IscS and SufS were labelled with ³⁵S in an ATP-independent manner (Figure 3Aa, b, lanes 4 and 8), suggesting the formation of an enzyme-bound persulphide (R-SSH). TtuB was ³⁵S-labelled only when incubated with a cysteine desulphurase and TtuC in the presence of ATP (Figure 3Ab, lanes 7 and 11). The conserved C-terminal glycine is the thiocarboxylation site in MoaD (Gutzke *et al*, 2001) and ThiS (Taylor *et al*, 1998). TtuB also has a glycine (Gly₆₅) at its C terminus

(Supplementary Figure S1A), and a Gly₆₅-deletion mutant of TtuB (TtuBΔG) was not ³⁵S-labelled (Figure 3B). These results suggest that cysteine is first activated by cysteine desulphurase as a persulphide form and the sulphur atom is then transferred to the C-terminal Gly₆₅ of TtuB by TtuC in an ATP-dependent manner.

Next, we further analysed the formation of TtuB-COSH by MS. TtuB was detected as a single peak with a mass of 7591.1 Da (Figure 4Aa), which is consistent with the theoretical value of 7591.50 Da. When TtuB was incubated with TtuC in the presence of ATP, a major peak (7920.4 Da) was observed together with several minor peaks (Figure 4Ab). The difference (329.2 Da) in mass between this peak and that of TtuB (7591.2 Da) corresponds exactly to that of an adenylate group, which indicates the formation of a TtuB acyladenylate (TtuB-COAMP). This annotation was further confirmed by MS/MS analysis (Figure 4B). A single peak corresponding to the hydrogen adduct of AMP (348.033 Da, theoretical mass 348.231 Da) was detected from the [TtuB-COAMP + 7H]⁷⁺ ion (*m/z* = 1132.5). When TtuB was incubated with TtuC and IscS or SufS in the presence of ATP and cysteine, a new 7607.1 Da peak was observed (Figure 4Ac, d). The increase of 15.9 Da from the mass of TtuB (7591.2 Da) can be interpreted as substitution of the oxygen atom with sulphur (16.0 Da, theoretical), suggesting the formation of a thiocarboxyl group at the C-terminal glycine of TtuB. These results indicate that the C-terminal glycine residue of TtuB is first activated by TtuC as an acyl-adenylate, and then thiocarboxylated by cysteine desulphurase and cysteine.

A thioester complex is formed between TtuB-Gly₆₅ and TtuC-Cys₁₉₂

When the reaction products of TtuB and TtuC were analysed by SDS-PAGE, a faint band was detected in a higher molecular weight position (Figure 3Ab, lane 3, with asterisk). This band was attributed to a covalent complex of TtuB and TtuC; however, only a small signal for the complex was detected by MS analysis (data not shown), probably because of the instability of the complex. Therefore, we changed the reaction conditions by lowering the incubation temperature from 65 to 30°C and increasing the incubation time from 15 min to 1 h. Under these conditions, the amounts of the complex increased to half of the input proteins (Figure 5B, lane 1). By MS analysis, TtuC was detected at a molecular weight of 31 659.3 Da, which corresponds to the N-terminal (Met₁)-truncated form, whose theoretical mass is 31 659.2 Da (Figure 5Aa, left panel). Truncation of the protein expressed in *E. coli* by methionyl-aminopeptidase has been previously reported (Matthies *et al*, 2005). Under these reaction conditions, we obtained an additional 39 233.7 Da peak (Figure 5Aa, left panel), which is attributed to the thioester complex of TtuB–TtuC (theoretical value 39 232.7 Da; TtuB + TtuC – H₂O). The remaining TtuB was detected as a major peak at 7651.9 Da and the acyladenylate form at 7921.0 Da (Figure 5Aa, right panel). The 7651.9 Da peak (TtuB + 60 Da) may be a thioester adduct of TtuB with 2-mercaptoethanol (β-ME) derived from the enzyme solution (theoretical value 7651.62 Da; TtuB + β-ME – H₂O).

To confirm that the TtuB–TtuC complex is formed by a thioester bond, reaction mixtures were treated with 20 mM dithiothreitol (DTT) at 40°C for 30 min. Upon reduction by DTT, the band for the TtuB–TtuC thioester disappeared, and

Figure 2 Characterization of the *ttc0979* mutant strain. (A) Schematic representation of TTC0979 (TtuC of *T. thermophilus*) sequence. The P-loop motif and the conserved cysteine residues are numbered. The mutation positions of variants (C192S and C268S) used in this study are also indicated. ThiF and MoeB from *E. coli* (EC) and *B. subtilis* (BS) are presented for comparison. The conserved amino acids are shaded. (B) Analysis of modified bases in tRNA hydrolysates (20 µg) by HPLC-MS. UV traces for *T. thermophilus* HB27 (wild type, upper panel) and NS2730 (*ttc0979::km*, lower panel) are shown. The modified bases were identified by their *m/z* and retention times: Ψ, pseudouridine; m¹A, 1-methyladenosine; m⁷G, 7-methylguanosine; m¹G, 1-methylguanosine; Gm, 2'-O-methylguanosine; m²G, 2-methylguanosine; s⁴U, 4-thiouridine; s²T, 2-thioribothymidine; GmG, dinucleotide GmpG; t⁶A, 6-theronylcarbamoyladenine; rT, ribothymidine. (C) Growth phenotypes of HB27 (wild type) and s²T-deficient strains (NS2730 (*ttuC::km*), NS2710 (*ttuA::km*), and NS2720 (*ttuB::km*)). Cells were cultured in rich medium (30 µg/ml kanamycin was added to the mutant strains) at 70°C overnight. Diluted cultures (*A*₆₀₀ = 0.01) and serial dilutions of these (10⁻¹, 10⁻², and 10⁻³) were spotted onto rich medium plates and incubated for 23 h at 60°C, 14 h at 70°C, 14 h at 75°C, 14 h at 80°C, or 21.5 h at 82°C.

bands corresponding to the intact TtuC and a new band near the position of TtuB appeared (Figure 5Bb, lane 9). By MS analysis, the 39 234 Da peak almost completely disappeared and TtuC (31 660.9 Da) was regenerated (Figure 5Ab, left

panel). A 7727.8 Da species (TtuB + 136 Da) was detected as the major peak for TtuB (Figure 5Ab, right panel), which is thought to correspond to a TtuB-DTT thioester adduct (theoretical value 7727.74 Da; TtuB + DTT-H₂O). The band

Figure 3 *In vitro* sulphur transfer to TtuB using recombinant proteins. Reaction mixtures were analysed by non-reducing 10–20% SDS–PAGE, stained with CBB (upper panels), and the ^{35}S radioactivity was visualized using phosphor imaging plates (lower panels). **(A)** Sulphur transfer to TtuB by TtuC and IscS or SufS: lane 1, TtuB; lane 2, TtuC; lane 3, TtuB and TtuC; lane 4, IscS; lane 5, TtuB and IscS; lane 6, TtuC and IscS; lane 7, TtuB, TtuC, and IscS; lane 8, SufS; lane 9, TtuB and SufS; lane 10, TtuC and SufS; lane 11, TtuB, TtuC, and SufS. The reaction was performed in the absence (a) or presence (b) of ATP. A faint band of the TtuB–TtuC thioester is indicated by an asterisk (*) in lane 3 of CBB-stained gel (b). **(B)** Sulphur transfer to the TtuBAG mutant was tested: lane 1, TtuB (wild type), TtuC, and IscS; lane 2, TtuBAG, TtuC, and IscS; lane 3, TtuB (wild type), TtuC, and SufS; lane 4, TtuBAG, TtuC, and SufS.

below the position of the intact TtuB (Figure 5Bb, lane 9) may correspond to this TtuB–DTT adduct. Adducts of sulphur-carrier proteins have also been detected in thiamin synthesis (Lauhon and Kambampati, 2000). Considerable amounts of ThiS + 60 Da and ThiS + 136 Da adducts were produced in an *in vitro* reaction with IscS and ThiF (a TtuC homologue). These adducts appear to correspond to β -ME and DTT adducts, respectively.

We further characterized the formation of the TtuB-TtuC thioester complex (Figure 5B). First, the complex formation was ATP dependent (lanes 1 and 5). Next, we determined the TtuB and TtuC residues that are required for thioester formation. The TtuBAG mutant was unable to form a complex (lane 2). A TtuB-DTT adduct was also formed after adenylation of TtuB (lanes 9 and 13), and the C-terminal glycine was the linkage site (lanes 9 and 10). The β -ME adduct may be formed by the same mechanism as the DTT adduct. Among six cysteine residues in the TtuC of *T. thermophilus* (Figure 2A), four cysteines (Cys₁₇₅, Cys₁₇₈, Cys₂₄₉, and Cys₂₅₂) are conserved and most probably bind a Zn²⁺ ion, similar to the cases for ThiF and MoeB (Lake *et al*, 2001; Duda *et al*, 2005). The other two cysteines are the conserved Cys₁₉₂ and semiconserved Cys₂₆₈. Therefore, we tested serine mutants of Cys₁₉₂ (C192S) and Cys₂₆₈ (C268S) for thioester formation. The C268S mutant was able to form a thioester (Figure 5B, lane 4), but the C192S mutant was not (lane 3). We therefore concluded that the carboxyl group of the C-terminal glycine (Gly₆₅) of TtuB is first adenylylated by TtuC and then the sulphhydryl group of the TtuC Cys₁₉₂ forms thioester bond. To our knowledge, this is the first evidence that a protein thioester is formed in a prokaryotic sulphur activation system.

TtuB-COSH is an intermediate sulphur donor for s²T formation

In our previous work, the *in vitro* formation of s²T in tRNAs was observed by incubation of a substrate tRNA having an rT54 (yeast tRNA^{Phe}) with TtuA, TtuB, cysteine desulphurase (IscS or SufS), and the desalted cell extract of *T. thermophilus* in the presence of ³⁵S-cysteine and ATP (Shigi *et al.*, 2006b). Here, we attempted to reconstitute s²T formation by substituting TtuC for the cell extract; however, no thiolation of tRNA was observed (data not shown). In addition, the enhancement of s²T formation by TtuC was not observed, even when small amounts of cell extract were used (data not shown). These results suggest that additional factors are required for s²T formation.

We next examined the involvement of the C-terminal glycine in s²T formation. Yeast tRNA^{Phe} was incubated with TtuA, TtuB, cysteine desulphurase, and a small amount of the desalted cell extract in the presence of ³⁵S-cysteine and ATP. After the reaction, RNAs were separated by denaturing PAGE, and incorporation of ³⁵S sulphur into tRNA^{Phe} was detected and quantified (Figure 6A and B). The faint band detected in a toluidine blue stained gel (lane 1) is the tRNAs derived from the *T. thermophilus* cell extract. These tRNAs were not good substrates for 2-thiolation, as compared with exogenously added yeast tRNA^{Phe} (Shigi *et al*, 2006b). In the presence of the desalted cell extract, s²T was formed by TtuA, TtuB, and cysteine desulphurase (lanes 4 and 7). In the presence of TtuBAG, minimal amounts of s²T were formed (lanes 5 and 8), similar to levels detected in the absence of TtuB (lanes 3 and 6). These activities may be attributed to TtuB present in the cell extract. This result suggests that the C-terminal thiocarboxyl group of TtuB is required for s²T synthesis.

Figure 4 Characterization of TtuB-COSH formation by MS. (A) The mass spectra of TtuB and its derivatives are shown. Theoretical values (a.m.u.) are indicated in parentheses (top). (a) Mass spectrum of TtuB before reaction. (b) Mass spectrum of TtuB incubated with TtuC in the presence of ATP. (c) Mass spectrum of TtuB incubated with TtuC and IscS in the presence of ATP and cysteine. (d) SufS was substituted for IscS in (c). (B) MS/MS spectrum generated from TtuB-COAMP in the reaction mixture of Ab.

We investigated more directly whether TtuB-COSH serves as a sulphur donor. We tested s^2T formation with TtuB-CO ^{35}S H instead of ^{35}S -cysteine. TtuB-CO ^{35}S H was formed by TtuC and SufS in the presence of ^{35}S -cysteine and ATP (Figure 6C, lane 1). After the reaction, His $_6$ -tagged TtuC and SufS were removed by Ni $^{2+}$ -affinity chromatography (lane 2), and the unreacted free ^{35}S -cysteine was removed by gel-filtration (lane 3). The efficiency of the thiocarboxylate formation was $\sim 20\%$.

Using purified TtuB-CO ^{35}S H as the sulphur donor, incorporation of ^{35}S to the substrate tRNA was assayed using TtuA and the desalted cell extract in the presence of ATP (Figure 6D). The sulphur atom of TtuB-CO ^{35}S H was incorporated into tRNA (lane 4). The reaction was dependent on both TtuA (lanes 4 and 6) and the desalted cell extract (lanes 4 and 8). Approximately 8% of the sulphur atoms in the input TtuB-CO ^{35}S H was transferred to the substrate tRNA, and TtuA enhanced the sulphur transfer nine-fold (Figure 6E). These results clearly show that TtuB can function as a sulphur carrier in s^2T synthesis. The unidentified factor(s) appear to be involved in transfer of the sulphur atom from TtuB-COSH to tRNA.

TtuC is involved in the biosynthesis of other sulphur compounds

A search of the *T. thermophilus* HB27 genome revealed that four (putative) sulphur carriers, TTC0105 (TtuB), TTC0316

(This), TTC1947 (MoaD for Moco synthesis), and TTC1835 (MoaD for tungsten cofactor (Wco) synthesis) (Supplementary Figure S1A), are present. Wco is a Moco-related cofactor, in which a tungsten atom is coordinated by a molybdopterin (Figure 1B), although Wco biosynthesis has not yet been elucidated in detail (Chan *et al*, 1995). As there is one set of molybdopterin biosynthesis genes (*moaA*, *moaC*, and *moaE*) in the *T. thermophilus* genome, TTC1947 and TTC1835 may function as sulphur carriers in Moco and Wco synthesis, respectively.

In contrast to these four putative sulphur carriers, there exists only one gene (*ttc0979 = ttuC*) that is homologous to *moeb/thiF* in the *T. thermophilus* genome, although other genes for Moco/Wco and thiamin biosynthesis are encoded in the genome (data not shown). We therefore hypothesized that, in *T. thermophilus*, TtuC is also involved in Moco/Wco and thiamin biosynthesis through activation of these specific sulphur carriers. Thus, we examined Moco and thiamin biosynthesis in the *ttuC* mutant strain.

To test the requirements for thiazole and thiamin for growth, the wild-type strain (HB27) and a *ttuC* mutant (NS2730) were cultured in minimal medium (Figure 7A). The wild-type strain grew without HET or thiamin. In contrast, NS2730 could not grow in minimal medium, but grew in minimal medium supplemented with HET or thiamin. This

Figure 5 Characterization of the TtuB-TtuC thioester. **(A)** The formation and scission of the TtuB-TtuC thioester were analysed by MS. The mass regions of 30 000–40 000 and 7500–8000 a.m.u. are shown in left and right panels, respectively. Theoretical mass values are indicated in parentheses (top). In **(b)**, the reaction mixture was further incubated with 20 mM DTT. **(B)** Analysis of the site of thioester bond formation. Reaction mixtures were analysed by non-reducing 10–20% SDS-PAGE and stained with CBB. Lanes 1, 5, 9, and 13: wild-type TtuB and TtuC; lanes 2, 6, 10, and 14: TtuBΔG and wild-type TtuC; lanes 3, 7, 11, and 15: wild-type TtuB and TtuC (C192S); lanes 4, 8, 12, and 16: wild-type TtuB and TtuC (C268S). Reactions were performed in the presence (lanes 1–4 and 9–12) or absence (lanes 5–8 and 13–16) of ATP. In **(b)**, the reaction mixtures were further treated with 20 mM DTT before electrophoresis.

result suggests that the mutant strain is unable to synthesize thiamin. The growth of the mutant strain was fully complemented by the addition of thiamin, but only partially complemented by HET. One possible explanation is that there is no apparent homologue of *thiM* (HET kinase) in the *T. thermophilus* genome. HET monophosphate (HET-P) is an intermediate in thiamin synthesis and HET can be used only after its conversion to HET-P by a salvage enzyme, the HET kinase (Petersen and Downs, 1997).

Moco is used in nitrate reductase, and the activity of nitrate reductase can thus be used to monitor Moco synthesis (Johnson and Rajagopalan, 1987). Respiratory nitrate reductase catalyses the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) in anaerobic respiration (Moreno-Vivián *et al*, 1999). The NAR1 strain of *T. thermophilus* possesses a nitrate reductase operon, and can therefore grow anaerobically by nitrate reduction (Ramírez-Arcos *et al*, 1998; Cava *et al*, 2007). Thus, we constructed a *ttuC* mutant (NS0130) from the NAR1 strain to analyse Moco biosynthesis. We confirmed

the absence of s^2T in NS0130 tRNAs by HPLC analysis (data not shown).

First, the growth phenotype was analysed under the previously developed ‘microaerobic’ conditions (Ramírez-Arcos *et al*, 1998). As shown in Figure 7B, wild-type NAR1 could grow anaerobically with the addition of nitrate, but NS0130 could not. Secondly, we examined the nitrate reductase activity by measuring the nitrite concentration of the culture medium (Table I). Nitrite production by wild-type cells was induced by nitrate, as reported previously (Ramírez-Arcos *et al*, 1998). However, no nitrite was produced by NS0130. These data suggest that, in the *ttuC* mutant, Moco was not synthesized and nitrate reductase activity was thereby abolished. In conclusion, *ttuC* is involved in three biosynthetic pathways in *T. thermophilus*, Moco and thiamin biosynthesis, as well as thiomodification of s^2T in tRNAs.

We next examined thiocarboxylate formation *in vitro* using recombinant TTC0316, TTC1947N, and TTC1835N proteins. For TTC1947 and TTC1835, the N-terminal domain

Figure 6 *In vitro* formation of $s^2\text{T}$ on tRNA. (A) $s^2\text{T}$ formation from cysteine. The reacted RNA (*S. cerevisiae* tRNA^{Phe}) was separated on 10% denaturing PAGE, and stained with toluidine blue (TB; upper panel). The ^{35}S radioactivity was visualized using a phosphor imaging plate (IP; lower panel). In TB stain, mainly SctRNA^{Phe} was visualized compared with low background bands of tRNAs derived from the cell extract. Desalted cell extract was added to all reactions. Lane 1, no additive; lane 2, tRNA only; lane 3, tRNA, TtuA, and IscS; lane 4, tRNA, TtuB, TtuA, and IscS; lane 5, tRNA, TtuBAG, TtuA, and IscS; lane 6, tRNA, TtuA, and SufS; lane 7, tRNA, TtuB, TtuA, and SufS; lane 8, tRNA, TtuBAG, TtuA, and SufS. (B) The assays in (A) were repeated in triplicate and the incorporation of ^{35}S to the substrate tRNA was quantified. The value of tRNA thiolation in lane 7 was set as 100%. (C) Preparation of ^{35}S -labelled TtuB-COSH. The reaction mixture after the incubation (lane 1), the flow-through fraction from Ni $^{2+}$ -NTA column (lane 2), and the gel-filtration sample (lane 3) were separated by non-reducing 10–20% SDS-PAGE. The gel was stained with CBB (left panel), and the ^{35}S radioactivity was visualized (right panel). (D) $s^2\text{T}$ formation on tRNA (*S. cerevisiae* tRNA^{Phe}) with TtuB-CO ^{35}SH . The reacted RNA was separated by 10% denaturing PAGE and stained with toluidine blue (TB; upper panel), and the ^{35}S radioactivity was visualized (IP; lower panel). In TB staining, SctRNA^{Phe} is the major component visualized compared with tRNAs derived from the cell extract. SctRNA^{Phe} was added to reactions in lanes 2, 4, 6, and 8; TtuA was added in lanes 3, 4, 7, and 8; the cell extract was added in lanes 3–6. (E) The assays in (D) were repeated in triplicate, and the incorporation of ^{35}S into tRNA was quantified. Only the values of lanes 4 (+ TtuA) and 6 (– TtuA) are presented. The sulphur-transfer values were calculated under the assumption that transfer of all the ^{35}S in the input TtuB-COSH to tRNA equals 100% transfer.

corresponding to sulphur-carrier protein was prepared. These three sulphur carriers were ^{35}S -labeled in a TtuC-dependent manner, as was TtuB (Supplementary Figure S2A). The sulphur transfer was dependent on the addition of ATP

Figure 7 The biosynthesis of other sulphur compounds in *ttuC* mutants. (A) Thiamin auxotroph. *T. thermophilus* HB27 (wild type) and NS2730 (*ttuC::km*) were grown in minimal medium supplemented with hydroxyethylthiazole (HET) or thiamin. Open triangle (wild type, no additive), open square (wild type, with HET), open circle (wild type, with thiamin), filled triangle (*ttuC::km*, no additive), filled square (*ttuC::km*, with HET), filled circle (*ttuC::km*, with thiamin). (B) Molybdenum cofactor deficiency. *T. thermophilus* NAR1 (wild type, open square) and NS0130 (*ttuC::km*, filled circle) were grown anaerobically in rich medium supplemented with (upper panel) and without (lower panel) 20 mM KNO $_3$.

Table I Production of nitrite in the culture medium

Strain	Nitrate	Nitrite produced
NAR1 (wt)	–	14.1 ± 0.7 μM
NAR1 (wt)	+ ^a	26.8 ± 3.8 mM
NS0130 (<i>ttuC::km</i>)	–	< 2 μM ^b
NS0130 (<i>ttuC::km</i>)	+ ^a	< 2 μM ^b

^aNitrate (40 mM) was supplemented in the culture medium.

^bDetection limit.

(data not shown). The efficiencies of the sulphur transfer to the four sulphur carriers were similar (Supplementary Figure S2B). We also confirmed the formation of adenylyl-intermediates and thiocarboxylated products by MS (Supplementary Table SI). These results further show that TtuC can activate all four sulphur carriers in *T. thermophilus*.

Discussion

Thiomodifications of uridine, in which sulphur is substituted for oxygen, occur in almost all organisms, and these simple modifications require multiple sulphur transfer reactions (Kessler, 2006). The sulphur atom of cysteine is activated by cysteine desulphurases as an enzyme-bound persulphide. The sulphur atom of the persulphide is transferred to specific sulphur-carrier protein(s) and/or a modification enzyme, which then incorporates the sulphur atom into tRNA. Previously, only protein-persulphide has been identified as a sulphur carrier used in thiouridine synthesis. In the wobble position (position 34), tRNA^{Lys}, tRNA^{Glu}, and tRNA^{Gln} possess a 2-thiouridine (s^2U) derivative, which is essential for codon-recognition in many living organisms (Curran, 1998). In *E. coli*, Tus proteins sequentially transfer the sulphur atom, in persulphide form, from IscS to a modification enzyme, MnmA (Ikeuchi *et al*, 2006). The 4-thiouridine synthesis in bacteria also uses a persulphide carrier containing a rhodanese domain (Palenchar *et al*, 2000). In contrast to these persulphide carriers, we have demonstrated here that *T. thermophilus* possesses a distinct type of activated sulphur used in thiouridine synthesis. Although the differences in the chemical properties between persulphide and thiocarboxylate remain to be clarified, these sulphur-carrier proteins may have evolved to deliver reactive sulphur atoms to specific targets and avoid nonspecific transfer of activated sulphur atoms, which could inactivate other biomolecules.

The putative s^2T biosynthetic pathway identified here is presented in Figure 8. A cysteine desulphurase (IscS or SufS) activates the sulphur atom of cysteine and forms an enzyme-bound persulphide (R-SSH). The C-terminal glycine (Gly₆₅) of the sulphur-carrier protein TtuB is first activated by an ATPase, TtuC, resulting in an acyl-adenylate (TtuB-COAMP), and then receives a sulphur atom from persulphide on the cysteine desulphurase to form thiocarboxylated TtuB (TtuB-COSH). TtuB can bind to TtuA (Shigi *et al*, 2006b). TtuA probably activates the tRNA target nucleoside by a mechanism similar to other tRNA modification enzymes possessing a PP-loop motif (Ikeuchi *et al*, 2005) and is directly involved in transfer of the sulphur atom from TtuB-COSH to tRNA. There may exist additional factors required in the last step of s^2T biosynthesis because sulphur transfer from TtuB-COSH to tRNA depends on the addition of a cell extract and the efficiency of tRNA thiolation is low (only 8%; Figure 6E), in contrast to the high efficiency of TtuB-COSH formation by purified factors (Figure 4Ac, d).

TtuA, which is involved in s^2T54 synthesis, belongs to a large protein family conserved across all domains of life (Jäger *et al*, 2004). Recently, another member of this family was identified as a gene required for the biosynthesis of s^2U34 in cytosolic tRNAs in eukaryotes, such as yeast and nematodes (Björk *et al*, 2007; Dewez *et al*, 2008). In contrast to the high sequence similarity (26% identical) between TtuA and a yeast homologue, Tuc1p, the difference in the target positions is intriguing. The unidentified factors for s^2T synthesis may have important functions in recognition of the target nucleoside by TtuA. The biosynthesis of s^2U in yeast cytosolic tRNA is an iron sulphur (Fe-S) protein-dependent pathway (Nakai *et al*, 2007), implying that Fe-S protein(s) may be involved in s^2T synthesis in *T. thermophilus*. TtuA is a candidate for a Fe-S protein because it possesses many

Figure 8 Schematic diagram showing the putative pathway for biosynthesis of s^2T . TtuB works as a sulphur carrier in s^2T synthesis and can form a thioester linkage with TtuC. See text for details.

conserved cysteines. By identifying the missing factor(s) and elucidating the involvement of a Fe-S protein, we will be able to develop a fully defined *in vitro* reconstitution system for s^2T synthesis and elucidate in more detail the chemistry of sulphur transfer and the control of s^2T content in thermophiles. These studies may also lead to an understanding of the biosynthesis of s^2U34 in eukaryotes.

Sulphur activation systems that use a protein-thiocarboxylate intermediate are widespread in biosynthesis of sulphur-containing biomolecules other than molybdenum cofactor (Moco) and thiamin, namely siderophores in *Pseudomonas* (Lewis *et al*, 2000; Godert *et al*, 2007) and cysteine in *Mycobacterium tuberculosis* (Burns *et al*, 2005). In this report, we demonstrated that 2-thiouridine biosynthesis of tRNA also uses a protein-thiocarboxylate and that a required ATPase, TtuC, functions in several biosynthetic pathways, including those for thiamin, Moco, and possibly Wco, as well as in thio-modification, in *T. thermophilus* (Figures 2 and 7, and Table I). This observation implies that several biosynthetic pathways of these sulphur-containing compounds have evolved from a common ancestor.

The mechanism by which *T. thermophilus* TtuC can recognize four distinct substrate proteins (Supplementary Figure S2 and Supplementary Table SI) is an interesting issue in enzyme-substrate recognition. The interactions between adenylation enzymes and their corresponding sulphur-carrier proteins have been analysed by crystallographic studies of the MoeB/MoaD and ThiF/ThiS complexes (Lake *et al*, 2001) (Lehmann *et al*, 2006). MoeB and ThiF have very similar tertiary structures, and the mechanisms of substrate recognition are also similar. The MoeB and ThiF surfaces formed by the P-loop, the loop before $\alpha 7$, $\beta 5$ – $\beta 8$, and $\alpha 8$, interact with the MoaD and ThiS surfaces, respectively, formed by $\beta 3$, $\beta 4$, and 7 residues from the C-terminal Gly, and these interactions are mostly hydrophobic (Supplementary Figures S1A, B and S3A). The ThiS_{310A} also interacts with ThiF. In addition, salt bridges are formed between MoaD R11 and MoeB D227, and between ThiS D7 and ThiF R230. Although not visible in these complex structures, the length and the sequence of the flexible 'crossover-loop' (Supplementary Figure S1B), which may clamp the tail of ThiS, are important for the adenylation reaction (Duda *et al*, 2005).

Considering the high level of sequence conservation between TtuC and *E. coli* ThiF/MoeB (Supplementary Figure S1B), and comparing the tertiary structure of the *E. coli* ThiF/MoeB and the homology model of TtuC (data not shown), a high level of backbone structural homology appears to exist between these ATPases. Thus, TtuC is predicted to recognize its four substrates in essentially the same manner as MoeB/ThiF recognizes Moad/ThiS, as described above. Although the sequence conservation of the four paralogues (TtuB, TTC0316, TTC1835N, and TTC1947N) is not very high, the four proteins seem to have similar topologies, as apparent in the structures of TTC1835N and TTC0316 and the homology models of TTC1947N and TtuB (Supplementary Figures S1A and S3B). Considering the sequence alignment of sulphur-carrier proteins on the basis of their tertiary structures (Supplementary Figure S1A) and the interactions observed in the homologous *E. coli* complexes described above, TtuC may bind the four sulphur carriers through the hydrophobic surfaces formed by the relatively conserved β 3 and the seven C-terminal residues of these proteins. Intriguingly, salt bridges could be formed between TtuC and each of the sulphur carriers, R12 of TTC1947N and R11 of TTC1835N to D232 of TtuC; E9 of TtuB and E7 of TTC0316 to R239 of TtuC (Supplementary Figure S3B). In contrast to these common characteristics of the four sulphur carriers, the sequence conservation of the residues of these four proteins corresponding to the β 4/3₁₀A regions of *E. coli* ThiS is relatively low, although these regions may form the TtuC recognition site. Another possible reason for the recognition of multiple substrates and thioester formation by TtuC is that the sequence and length of the crossover loop of TtuC differ from those of MoeB/ThiF (Supplementary Figure S1B). In addition, the longer C-terminal extension (E255-R271) of TtuC may enable interactions with multiple sulphur carriers. Crystallographic structure determination and other empirical evidence will be needed to firmly determine the protein-protein interaction surfaces.

The structural and biochemical similarities suggest that an evolutionary relationship between these sulphur-activating systems and the eukaryote-specific protein-modification systems may exist (Hochstrasser, 2000) (Figure 1C). The sulphur-carrier proteins, Moad, ThiS, and TtuB, possess conserved glycines in their C-terminal ends, similar to ubiquitin and the ubiquitin-like modifier (Ub/Ubl). Moad (Lake *et al*, 2001) and ThiS (Wang *et al*, 2001), as well as *T. thermophilus* TtuB (Homology model, see Supplementary Figure S3), consist of a ubiquitin/ β -grasp fold, although the amino-acid conservation with Ub/Ubl is rather low. MoeB (Lake *et al*, 2001) and ThiF (Duda *et al*, 2005) have sequences and structures closely related to the Ub/Ubl-activating enzymes (E1). These ATPases catalyse adenylation of sulphur carriers or Ub/Ubl. After adenylation, Ub/Ubl is covalently attached to a cysteine residue in E1 through a thioester bond. However, a thioester intermediate has never before been detected in the biosynthesis of sulphur cofactors. A recent report has shown that a similar conjugate was formed in the *E. coli* thiamin biosynthesis pathway: ThiS is covalently linked to a conserved cysteine in ThiF (equivalent to Cys₁₉₂ of TtuC of *T. thermophilus*) through an acyl-disulphide linkage (ThiS-CO-S-S-ThiF) (Figure 1Cb) (Xi *et al*, 2001), although the conjugate was not detected in the *Bacillus subtilis* pathway (Park *et al*, 2003).

Surprisingly, the C-terminal acyladenylate of TtuB reacted with the sulphhydryl group of Cys₁₉₂ of TtuC, and a TtuB-TtuC thioester was formed *in vitro* (Figures 5 and 8). To our knowledge, this is the first protein thioester detected in sulphur activation machineries. In contrast to the acyl-disulphide complex in thiamin biosynthesis, the TtuB-TtuC complex takes exactly the same chemical form as the Ub/Ubl-E1 thioester. The other three sulphur carriers also formed thioesters with TtuC *in vitro* (Supplementary Figure S4 and Supplementary Table SI). These results would support the hypothesis that the eukaryotic Ub/Ubl conjugation system and the sulphur compound biosynthesis systems have evolved from a common ancestral system (Hochstrasser, 2000), although the *in vivo* existence and function of these thioesters should be clarified. Our observations may also suggest the novel possibility that thioester intermediates and isopeptide conjugates are formed in bacteria and archaea (Figure 8), although ubiquitin conjugates have been observed only in eukaryotes at this point. In support of this hypothesis, Ub-, E1-, and E2-related proteins have been found in genomes of several bacteria (Iyer *et al*, 2006). The s²T biosynthetic proteins may be the target of TtuB conjugation, and s²T biosynthesis may be regulated by TtuB conjugation.

Intriguingly, our results suggest that the formation of the TtuB-TtuC thioester is not directly involved in s²T synthesis, according to the following observations. (1) TtuB-COSH was the major product and minimal amounts of TtuB-TtuC thioester were detected in reaction of TtuB and TtuC with cysteine desulphurase in the presence of cysteine and ATP (Figure 4Ac, d). (2) The formation of a TtuB-TtuC thioester appears to be nonessential for TtuB-COSH formation *in vitro* because TtuC (C192S) could catalyse the formation of TtuB-COSH (Supplementary Figure S5). (3) Purified TtuB-COSH could be a sulphur donor for s²T synthesis (Figure 6). However, the exact function of the TtuB-TtuC thioester and its relationship to s²T synthesis *in vivo* need to be investigated in more detail.

Materials and methods

Strains and media

The strains used in this study are *T. thermophilus* HB27, NAR1 (gift from Dr J Berenguer) (Cava *et al*, 2007), NS2710 (*ttuA::km*), NS2720 (*ttuB::km*) (Shigi *et al*, 2006b), and NS0801 (*thiI::km*) (Shigi *et al*, 2006a). Wild-type and mutant strains were cultured in rich medium (Takada *et al*, 1993) at 70°C in the absence and presence of 30 µg/ml kanamycin, respectively, unless otherwise stated.

Analysis of tRNA modification

The hydrolysates of tRNA from each strain were prepared and analysed using LC/MS as described previously (Shigi *et al*, 2006b). We used an HPLC system equipped with photo-diode-array detector (GL-Sciences) for analysis of the *ttuC::km* strain of NAR1 (NS0130).

In vitro assay for thiocarboxylate formation

Assays were performed at 65°C for 15 min in 20 µl of T buffer (50 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM Mg(OAc)₂, and 0.1 mM DTT) containing 1 mM ATP, 10 µM ³⁵S-cysteine (2 µCi, American Radiolabeled Chemicals), 20 µM pyridoxal 5'-phosphate (PLP), and recombinant proteins (each of the sulphur carriers (TtuB, TTC0316, TTC1947N, and TTC1835N; 100 pmol), TtuC (50 pmol), and IscS (50 pmol) or SufS (50 pmol)). Reaction was stopped by adding an equal volume of sample loading solution (50 mM Tris-HCl (pH 6.8), 2% SDS, 0.003% bromophenol blue, and 8% glycerol) to the reaction mixture. SDS-PAGE was performed using 10–20% acrylamide gels (SuperSep HG, Wako). The gels were

stained with CBB, dried, exposed on an imaging plate, and radioactivity was detected using a BAS 2500 system (Fuji Photo System).

In vitro characterization of the TtuC thioesters

For the detection of the TtuC thioesters, each of the sulphur carriers (100 pmol) was incubated with TtuC (50 pmol) at 30°C for 1 h in 10 µl of T buffer containing 1 mM ATP. To reduce the thioester bond, 20 mM DTT was added to the reaction mixture and further incubated at 40°C for 30 min. Reaction mixtures were analysed by nonreducing SDS-PAGE as described above.

MS analysis

For the detection of acyladenylate intermediates, each of the sulphur carriers (100 pmol) was incubated with TtuC (50 pmol) at 65°C for 1 h in 20 µl of T buffer containing 1 mM ATP. For the detection of thiocarboxylates, each of the sulphur carriers (100 pmol) was incubated with TtuC (50 pmol) and IscS or SufS (50 pmol) at 65°C for 1 h in 20 µl of T buffer containing 1 mM ATP, 10 µM cysteine, and 20 µM PLP. For characterization of the TtuC thioesters, the complexes were formed and reduced as described above. The reaction mixtures were analysed using an LC-ESI-Q-TOF mass spectrometer, Q-STAR XL (Applied Biosystems). Further details are provided in the Supplementary data.

In vitro s^2T formation of tRNA with ^{35}S -cysteine as a sulphur donor

s^2T formation on a substrate tRNA was assayed as described previously (Shigi *et al*, 2006b), with the following slight modifications. The assays were performed at 60°C for 20 min in 25 µl of H buffer (50 mM HEPES-KOH buffer (pH 7.6), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 0.2 mM phenylmethylsulphonyl fluoride) containing 4 mM ATP, 10 µM ^{35}S -cysteine (10 µCi), 20 µM PLP, 4 µg yeast tRNA^{Phe} (Sigma), 100 µg of desalted cell extract (from *T. thermophilus* NS0801 grown at 80°C), and 30 pmol each of the recombinant proteins (TtuA, TtuB, and IscS or SufS). TtuB without a His₆ tag was used in this study. After the reactions, RNA was recovered using the acid-guanidinium thiocyanate-phenol-chloroform reagent (Isogen, Wako) and precipitated with ethanol. The RNA samples were incubated at 37°C for 1 h in 100 mM HEPES-KOH (pH 9.0) to hydrolyse the aminoacylated tRNA^{Cys} that was originally present in the cell extract and that was cysteinylated during the assay. After this treatment, RNA was separated using PAGE with 10% gels containing 7 M urea, and the gels were then stained with 0.025% toluidine blue. Finally, the gels were dried, exposed on an imaging plate, and analysed using the BAS 2500 system.

In vitro s^2T formation of tRNA with TtuB-CO³⁵SH as a sulphur donor

For ^{35}S -labeling of TtuB, TtuB (3 nmol) was incubated with His₆-TtuC (0.375 nmol) and His₆-SufS (0.375 nmol) at 65°C for 20 min in 300 µl of T buffer containing 1 mM ATP, 20 µM ^{35}S -cysteine (60 µCi),

and 20 µM PLP. The reaction mixture was then mixed with 15 µl of Ni²⁺-NTA-agarose (Qiagen) to remove His₆-tagged TtuC and SufS, and the unreacted ^{35}S -cysteine was next removed using a NAP 5 gel filtration column (GE Healthcare) with H buffer containing 0.1 mM DTT. The sample was kept on ice before use.

Subsequently, tRNA 2-thiolation assays were performed at 60°C for 20 min in 40 µl of H buffer containing 4 mM ATP, 4 µg yeast tRNA^{Phe}, 150 µg of desalted NS0801 cell extract, 30 pmol of TtuA, and 12 µl of TtuB-CO³⁵SH (~6 pmol). The incorporation of ^{35}S into the substrate tRNA was detected, as described above, with the exception that the alkaline treatment was not performed.

Thiamin auxotroph

Overnight cultures were inoculated into minimal medium minus thiamin (MM-T) with or without 20 µg/ml HET or 2 µg/ml thiamin. MM-T was prepared exactly as the MM medium (Takada *et al*, 1993), except that thiamin was not added. The cultures were incubated at 70°C with shaking and the optical density at 600 nm was measured at various times.

Molybdenum cofactor deficiency

Growth in 'microaerobic' conditions was tested as described (Ramírez-Arcos *et al*, 1998). Overnight cultures were inoculated into 4 ml rich medium with 20 mM KNO₃ in 9 ml screw-capped tubes, filled with mineral oil to the top of the tubes, and incubated at 70°C without shaking. The optical density at 660 nm was measured at various times.

To measure the nitrate reductase activity, cells were grown in rich medium at 70°C with shaking up to an OD₆₀₀ of ~0.7. After addition of 40 mM KNO₃, cells were cultivated in capped tubes at 70°C without shaking for an additional 6 h. The nitrite concentration of the culture medium was then determined (Snell and Snell, 1949). Diluted supernatants (150 µl) were mixed with 150 µl of 1% sulphanilamide (in 2.5 M HCl) and 150 µl of 0.02% *N*-(1-naphthyl)-ethylenediamine dihydrochloride. After 30 min, the amount of nitrite was measured by absorbance at 540 nm. We used serial dilutions of NaNO₂ as a standard.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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