

Article

Transfer RNA Methyltransferases from *Thermoplasma acidophilum*, a Thermoacidophilic Archaeon

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Abstract: We investigated tRNA methyltransferase activities in crude cell extracts from the thermoacidophilic archaeon *Thermoplasma acidophilum*. We analyzed the modified nucleosides in native initiator and elongator tRNA^{Met}, predicted the candidate genes for the tRNA methyltransferases on the basis of the tRNA^{Met} and tRNA^{Leu} sequences, and characterized Trm5, Trm1 and Trm56 by purifying recombinant proteins. We found that the *Ta0997*, *Ta0931*, and *Ta0836* genes of *T. acidophilum* encode Trm1, Trm56 and Trm5, respectively. Initiator tRNA^{Met} from *T. acidophilum* strain HO-62 contained G⁺, m¹I, and m²₂G, which were not reported previously in this tRNA, and the m²G26 and m²₂G26 were formed by Trm1. In the case of elongator tRNA^{Met}, our analysis showed that the previously unidentified G modification at position 26 was a mixture of m²G and m²₂G, and that they were also generated by Trm1. Furthermore, purified Trm1 and Trm56 could methylate the precursor of elongator tRNA^{Met}, which has an intron at the canonical position. However, the speed of methyl-transfer by Trm56 to the precursor RNA was considerably slower than that to the mature transcript, which suggests that Trm56 acts mainly on the transcript after the intron has been removed. Moreover, cellular arrangements of the tRNA methyltransferases in *T. acidophilum* are discussed.

Keywords: RNA modification; tRNA methyltransferase; archaea

1. Introduction

Thermoplasma acidophilum is a thermoacidophilic archaeon that grows optimally at 59 °C and pH 1.9 [1]. The characteristic property of this archaeon is that the cells are very irregular in shape due to the lack of a cell wall [2,3]. Despite this, the cytoplasmic membrane tolerates an acidic environment at high temperatures. Consequently, components of the membrane have been studied in detail [4]. Furthermore, lipoylation of proteins [5], biosynthesis of lipids [6], cell surface glycoproteins [7] and a channel protein [8] have been also studied in *T. acidophilum*. Given that a prokaryotic histone-like DNA binding protein was discovered first from *T. acidophilum* [9,10], the bacterium has been used as a model system to investigate DNA replication, DNA repair, and transcriptional initiation in archaea [11–16]. Furthermore, the energy metabolism of *T. acidophilum* has been studied in detail because it can grow under an extreme microaerophilic environment [17,18]. Genome sequencing elucidated that the *T. acidophilum* genome encodes only approximately 1500 open reading frames [19]. Consequently, large protein complexes such as the proteasome and chaperonin are composed of a relatively limited number of protein subunits, and thus they have been studied and compared with their more complicated counterparts from eukaryotes [20–23].

Although *T. acidophilum* has been investigated from various viewpoints as described above, there is little knowledge about tRNA modifications, with the exception of some early studies [24–27] and our more recent work [28]. In 1981 and 1982, the sequences of the initiator ([25] and Figure 1A) and elongator tRNA^{Met} ([24] and Figure 1B) were determined. A novel modification at position 15 (N in Figure 1B), which was named later as archaeosine at position 15 (G⁺15) [29], and the typical archaeal tRNA modification of 2'-*O*-methylcytidine at position 56 (Cm56) [30] were reported. In 1991, Edmonds *et al.* [27] reported that a mixture of tRNAs from *T. acidophilum* contains *N*⁷-methylguanine (m⁷G). In general, the m⁷G modification is found at position 46 in class I tRNAs from eubacteria and eukaryotes [31–35]; class I tRNAs are defined as tRNAs with a variable region of regular size. To verify the location of the m⁷G modification in the tRNA, we analyzed tRNA modifications in *T. acidophilum* [28]. Unexpectedly, we found that the m⁷G modification was present at a novel position, nucleotide 49 in class II tRNA^{Leu} (Figure 1C); class II tRNAs have a long variable region. Furthermore, we found several distinct modifications in this tRNA^{Leu} (Figure 1C): 4-thiouridine at position 9 (s⁴U9) [36–39], G⁺13 [29,40], and 5-carbamoylmethyluridine at position 34 (ncm⁵U34) [41,42]. The modifications s⁴U9 and ncm⁵U34 have been not found in other archaeal tRNAs and G⁺13 has not been reported in any other tRNA [31,32]. In the current study, we tested the tRNA methyltransferase activities in crude cell extract from *T. acidophilum*, analyzed the methylated nucleosides in purified tRNAs and characterized the tRNA methyltransferases by expressing recombinant proteins in *Escherichia coli*.

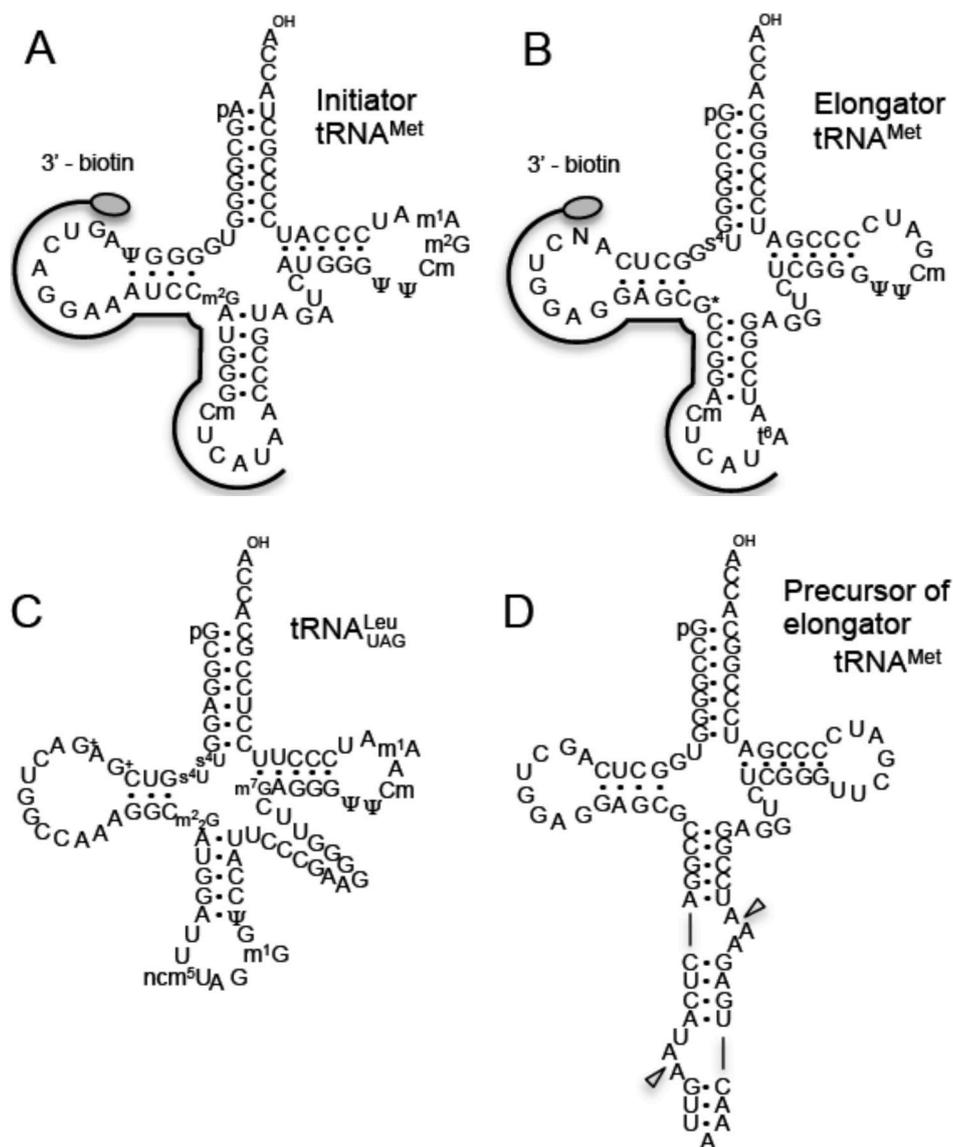


Figure 1. tRNAs and precursor tRNA from *T. acidophilum*. The sequences of initiator tRNA^{Met} (A), elongator tRNA^{Met} (B) and tRNA^{Leu}_{UAG} (C) are depicted by cloverleaf structures. The regions to which the 3'-biotinylated DNA probes hybridize are illustrated. The abbreviations are as follows: pseudouridine, ψ ; *N*²-methylguanosine, m²G; 2'-*O*-methylcytidine, Cm; 1-methyladenosine, m¹A; 4-thiouridine, s⁴U; unknown modification, N; unidentified G modification, G*; *N*⁶-thereonylcarbamoyladenine, t⁶A; archaeosine, G⁺; *N*²,*N*²-dimethylguanosine, m²G; 5-carbamoylmethyluridine, ncm⁵U; 1-methylguanosine, m¹G; 7-methylguanosine, m⁷G. The precursor of elongator tRNA^{Met} contains an intron at the canonical site (D). Triangles show the cleavage sites of tRNA splicing endonuclease.

2. Results and Discussion

2.1. Methylated Nucleosides in *T. acidophilum* tRNAs

Figure 1A–C show the cloverleaf structures of initiator tRNA^{Met}, elongator tRNA^{Met} and tRNA^{Leu}_{UAG}, respectively. In the current study, we utilized these tRNA sequences as a basis to characterize tRNA

methyltransferases in *T. acidophilum*. As shown in Table 1, various methylated nucleosides are present in these tRNAs. We predicted the candidate genes for the enzymes responsible for the modifications by BLAST searches [28] and analyzed the corresponding recombinant proteins. For example, given that the G26 in elongator tRNA^{Met} was reported to be a modified G (G*) ([24] and Figure 1B) we investigated whether recombinant Trm1 methylated G26 in the elongator tRNA^{Met} transcript. The results of the current study are summarized in Table 1. Furthermore, it should be mentioned that the sequence of the initiator tRNA^{Met} that is encoded in the genome of *T. acidophilum* strain HO-62 differs from that reported in the earlier study ([25] and Figure 1A): the nucleotide at position 57 is A instead of G in strain HO-62. In archaeal tRNAs, A57 is often modified to 1-methylinosine (m¹I57) via 1-methyladenosine (m¹A57) by TrmI and deamination [43–45]. Some possible explanations for this discrepancy are elaborated in the Discussion section. Moreover, in the case of the precursor of elongator tRNA^{Met}, a standard intron is inserted at the canonical position between nucleotides 38 and 39 (Figure 1D). Therefore, it is possible that the presence of the intron might affect the methylations by tRNA methyltransferases.

Table 1. Methylated nucleosides in *T. acidophilum* tRNAs.

Modification and Position	Candidate Enzyme and Gene		Results from This Study
Initiator tRNA^{Met}			
m ² G26	Trm1	<i>Ta0997</i>	m ² ₂ G26 and m ² G26 formed by Trm1.
Cm32	TrmJ?	<i>Ta1010m?</i>	¹ We did not analyze this gene product.
Cm56	Trm56	<i>Ta0931</i>	Cm56 formed by Trm56.
m ² G57	?		² This position in the tRNA gene is A57.
m ¹ A58	TrmI	<i>Ta0852?</i>	³ We could not detect m ¹ A58 formation activity in the recombinant protein.
Elongator tRNA^{Met}			
G*26	Trm1	<i>Ta0997</i>	m ² ₂ G26 and m ² G26 formed by Trm1.
Cm32	TrmJ?	<i>Ta1010m?</i>	¹ We did not analyze this gene product.
Cm56	Trm56	<i>Ta0931</i>	Cm56 formed by Trm56.
tRNA^{Leu}_{UAG}			
m ² ₂ G26	Trm1	<i>Ta0997</i>	m ² ₂ G26 formed by Trm1.
m ¹ G37	Trm5	<i>Ta0836</i>	m ¹ G37 formed by Trm5.
m ⁷ G49	?	<i>Ta0679? +α?</i>	We could not obtain soluble recombinant protein.
Cm56	Trm56	<i>Ta0931</i>	Cm56 formed by Trm56.
m ¹ A58	TrmI	<i>Ta0852?</i>	³ We could not detect m ¹ A58 formation activity in the recombinant protein.

¹ During the course of the current study, it has been reported that *Sulfolobus acidocaldarius* TrmJ generates the Cm32 modification in tRNA. The candidate gene in *T. acidophilum* was predicted by a BLAST search;

² The sequence of the initiator tRNA^{Met} that is encoded in the genome of the *T. acidophilum* strain HO-62 differs from that reported in the earlier study [25] (see Figures 1A and 6A): the nucleotide at position 57 is A instead of G in strain HO-62. Furthermore, the tRNA gene in the genome of strain HO-62 contains additional nucleotides, A20b and C22;

³ The *Ta0852* gene product was expressed in *Escherichia coli* as a soluble protein. However, we could not detect any ability to form m¹A58; ?, the corresponding enzyme is unknown.

2.2. Transfer RNA Methyltransferase Activities in the Crude Cell Extract

Next, we tested tRNA methyltransferase activities in crude extract from *T. acidophilum* cells. The supernatant fraction from centrifugation at 30,000× g (S-30) was prepared and then the tRNA^{Leu}_{UAG} transcript was subjected to methylation by the S-30 extract with ¹⁴C-S-adenosyl-L-methionine (AdoMet) as the methyl group donor. The methylated tRNA was digested completely with nuclease P1 and then the resultant ¹⁴C-methylated nucleotides were analyzed by two-dimensional thin-layer chromatography (2D-TLC). As shown in Figure 2A, four ¹⁴C-methylated nucleotides (pm¹G, pm²G, pm²₂G and pm⁶A) could be detected. On the basis of the sequence of tRNA^{Leu}_{UAG} (Figure 1C) and the candidate enzymes (Table 1), pm¹G, pm²G and pm²₂G, and pm⁶A were expected to be derived from the activities of Trm5, Trm1, and TrmI, respectively: pm⁶A could be converted from pm¹A non-enzymatically [46]. However, unexpectedly, pCm and pm⁷G were not detected. In general, the formation of pCm by Trm56 is one of the most common tRNA methyltransferase activities found in crude extract from archaeal cells. For example, it was reported that Trm56 activity in relation to the formation of pCm56 is clearly detected in cell extract from *Pyrococcus furiosus* [47]. Analysis of the *T. acidophilum* proteome revealed that various proteins form several large (more than 300 kDa) protein complexes and that some protein complexes might interact with the membrane [48]. Consequently, Trm56 and an unknown tRNA (m⁷G49) methyltransferase might be included in protein complexes and precipitated by centrifugation at 30,000× g. We tested several buffer conditions such as variations in pH, components, detergents, and salt concentrations (data not shown). However, to date, we have not detected enzyme activities responsible for the formation of pCm and pm⁷G in crude extract from *T. acidophilum* cells, though there are other possibilities; while we used aluminum oxide to prepare the extract in this experiment, other methods for preparation of cell extract should be tested. In addition, tRNA methyltransferases in the crude extract have different affinities for ¹⁴C-AdoMet. The concentration of ¹⁴C-AdoMet in the experiment was 19.5 μM. In this case, tRNA methyltransferases, which have relatively high affinity for AdoMet, might preferentially consume the ¹⁴C-AdoMet. When the supernatant fraction from centrifugation at 100,000× g (S-100) was used as the cell extract instead of the S-30 fraction, the findings were even more marked: only the formation of pm¹G was detectable (Figure 2B). Given that tRNA methyltransferases have a general affinity for RNA, the enzymes often bind to ribosomes and are precipitated by centrifugation at 100,000× g. In fact, the majority of TrmI from *Thermus thermophilus* [49] is precipitated by centrifugation at 100,000× g [50]. However, our findings with the extract from *T. acidophilum* are unprecedented. In the current study, we characterized tRNA methyltransferases by analyzing purified recombinant proteins. However, these enzymes might interact with other proteins and form large protein complexes in living cells.

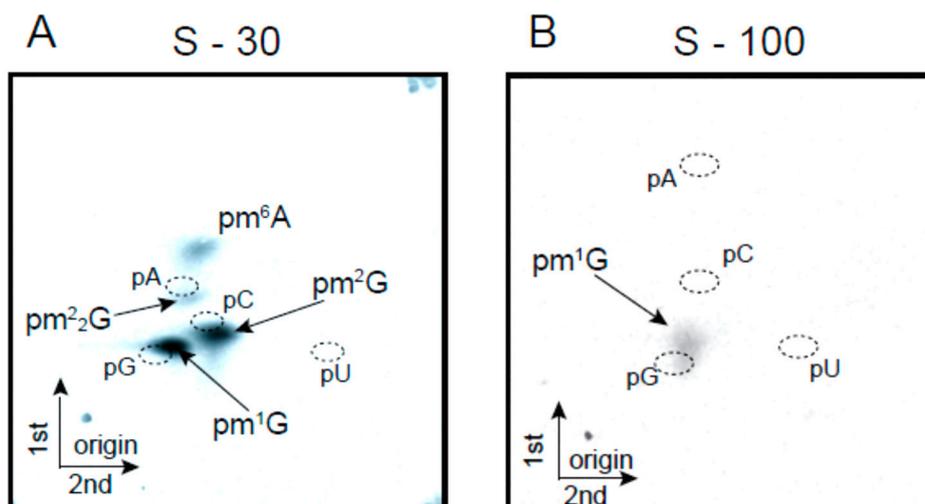


Figure 2. Activities of tRNA methyltransferases in the crude cell extracts. The activities of tRNA methyltransferases in the S-30 (A) and S-100 (B) fractions were analyzed by 2D-TLC. ^{14}C -methylated nucleotides were monitored by autoradiography. The solvent systems were as follows: first dimension, isobutyric acid, ammonia, water, 66/1/33 v/v/v; second dimension, isopropyl alcohol, HCl, water, 70/15/15 v/v/v.

2.3. Modified Nucleosides in Purified tRNAs

To analyze the modified nucleosides in tRNAs, we purified initiator and elongator tRNA^{Met} by the solid-phase DNA probe method [51], in which tetraalkylammonium salts were used in the hybridization buffer. Tetraalkylammonium salts destabilize the tRNA structure and enhance the formation of DNA-RNA hybrids [51], and thus we were able recently to purify single tRNA species from thermophiles such as *Aquifex aeolicus* [52], *T. thermophilus* [50,53], *Aeropyrum pernix* [54], and *T. acidophilum* [28] using this approach. In the case of initiator tRNA^{Met}, because the sequence from G15 to U36 was distinct, we designed the DNA probe to this region. In the case of elongator tRNA^{Met}, *T. acidophilum* two species: the sequence of one was previously determined as shown in Figure 1B [24]. These two tRNA^{Met} species differ in three nucleotides in the D-loop and anticodon arm. Therefore, we designed the DNA probe as shown in Figure 1B. As shown in the insets in Figure 3A,B, initiator and elongator tRNA^{Met} were purified successfully: the ^{14}C -Met-charging activities were checked by the S-100 fraction (data not shown). The purified tRNAs were digested with snake venom phosphodiesterase, RNase A and bacterial alkaline phosphatase, and then the resultant nucleosides were analyzed by HPLC using a C18 column (Figure 3A,B). Snake venom phosphodiesterase can cleave the phosphodiester bond adjacent to 2'-O-methylated nucleotide. As shown in Figure 3A, m¹A, Cm, m¹I, m²G, m²₂G and m⁶A were detected as methylated nucleosides in the initiator tRNA^{Met} sample. The presence of m¹A, Cm, m²G and m⁶A is consistent with the published RNA sequence (Figure 1A). However, the presence of m¹I suggests that A57 in this tRNA is modified to m¹A57 by TrmI [44,45] and that deamination then generates m¹I57 as in the case of *Haloferax volcanii* [43]. Furthermore, a peak for m²₂G was detected, which suggests that some proportion of the initiator tRNA^{Met} contains m²₂G26. Moreover, G⁺ was clearly detected, which suggests that G15 is modified to G⁺15 in initiator tRNA^{Met}. In the elongator tRNA^{Met} sample, m¹A, Cm, m²G, m²₂G and m⁶A were detected. The modifications

m^2G and m^2_2G have not been reported at any position in elongator $tRNA^{Met}$, although the uncharacterized G26 modification represents a possible location (G*26 in Figure 1B and Table 1). Consequently, the modified G26 was expected to be a mixture of m^2G26 and m^2_2G26 . Furthermore, N^6 -threonylcarbamoyladenine (t^6A) was also detected, which is consistent with the RNA sequence (Figure 1B). After these pilot experiments, we established expression systems in *E. coli* for the candidate genes shown in Table 1. As mentioned in Table 1, we could not obtain soluble protein from the *Ta0679* gene. Furthermore, the *Ta0852* gene product did not show TrmI activity. Consequently, these gene products were not analyzed further in the current study.

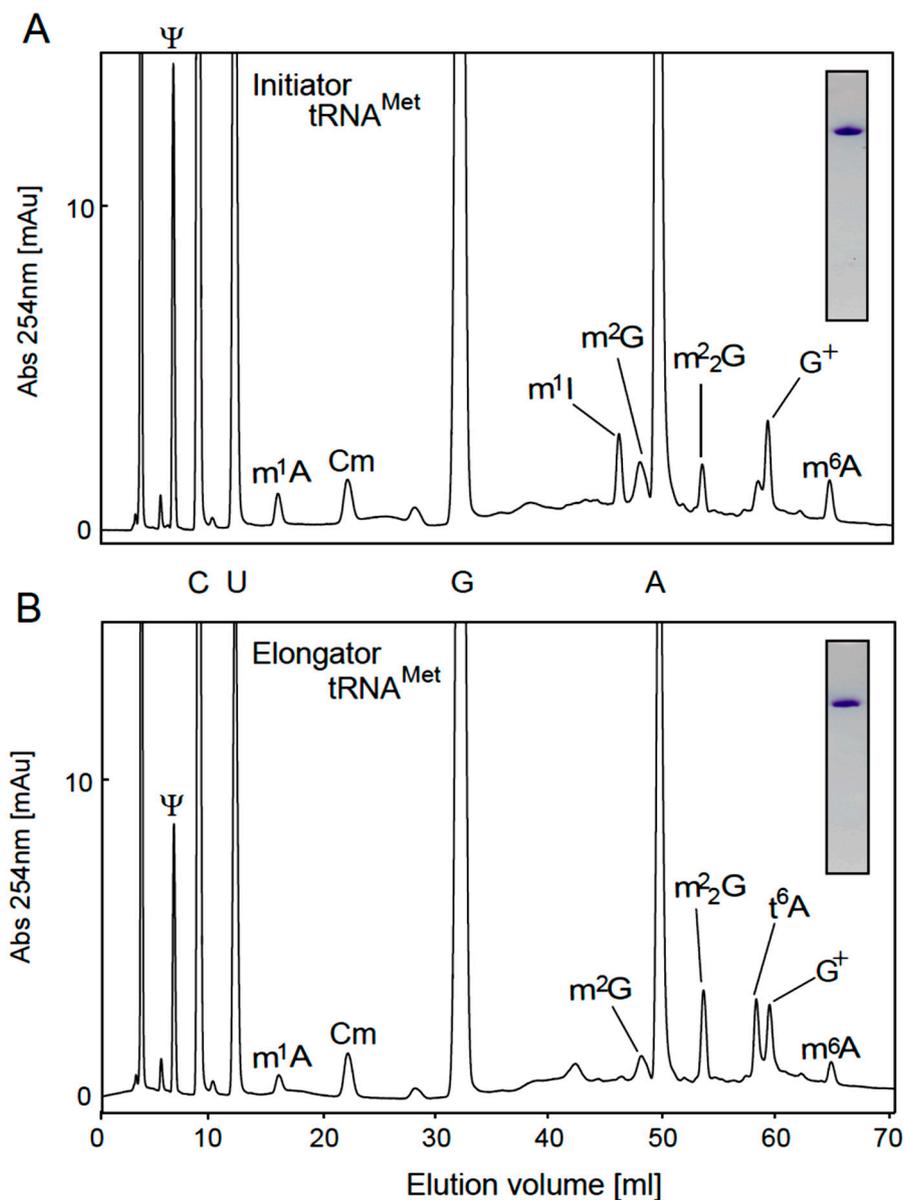


Figure 3. Modified nucleosides in native initiator and elongator $tRNA^{Met}$. Native initiator and elongator $tRNA^{Met}$ were purified by the solid-phase DNA probe method (insets). The hybridization regions of the probes are illustrated in Figure 1A,B. The modified nucleosides in the initiator (A) and elongator (B) $tRNA^{Met}$ were analyzed by reverse phase column chromatography.

2.4. Formation of m^1G37 in $tRNA^{Leu}_{UAG}$ Transcript by $Ta0836$ Gene Product

The $Ta0836$ gene product was expressed in *E. coli* and purified as shown in Figure 4A. The expected amino acid sequence of the $Ta0836$ gene product shares a high degree of homology (82%) with that of the identified archaeal Trm5 (Mj0883 of *Methanocaldococcus jannaschii*) [55,56]. When the purified protein was incubated with the $tRNA^{Leu}_{UAG}$ transcript and ^{14}C -AdoMet, the ^{14}C -methyl group was clearly incorporated into the transcript (see Figure 4C lane 1). Analysis of the modified nucleotides by 2D-TLC revealed that the ^{14}C -methylated nucleotide was pm^1G (Figure 4B). Furthermore, when the G37 in $tRNA^{Leu}_{UAG}$ transcript was replaced by A, no methyl group incorporation was observed (Figure 4C lane 2), which indicates that the methylation site is G37. From these results, we concluded that the $Ta0836$ gene product is the *T. acidophilum* Trm5 protein. Given that the m^1G modification was previously found only at position 37 in $tRNA^{Leu}_{UAG}$ in *T. acidophilum* ([28] and Figure 1C), the m^1G modification activity in the S-100 is probably derived from Trm5.

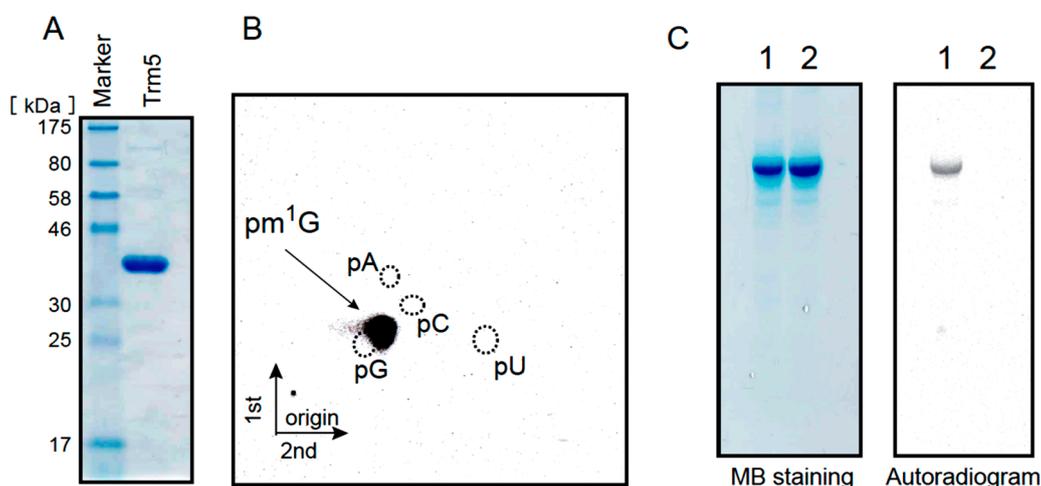


Figure 4. The $Ta0836$ gene product is *T. acidophilum* Trm5. (A) 10 μ g of the purified $Ta0836$ gene product was analyzed by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue; (B) The $tRNA^{Leu}_{UAG}$ transcript was methylated by the $Ta0836$ gene product and then the generated methylated nucleotide was analyzed by 2D-TLC; (C) The methyl group acceptance activities of the wild-type $tRNA^{Leu}_{UAG}$ transcript (lane 1) and the mutant $tRNA^{Leu}_{UAG}$ transcript (lane 2), in which G37 was replaced by A, were investigated. The transcripts were individually incubated with the $Ta0836$ gene product and ^{14}C -AdoMet, and then separated by 10% PAGE (7 M urea). The gel was stained with methylene blue (**left** panel) and the autoradiogram of the same gel was taken (**right** panel).

2.5. $Ta0997$ Gene Product Is a Single Site-Specific Trm1

The m^2G modification was observed only at position 26 in $tRNA^{Leu}_{UAG}$ ([28] and Figure 1C). Trm1 transfers two methyl groups to the 2-amino group in the target guanine and m^2G is formed as an intermediate [57,58]. Consequently, the m^2G and m^2_2G modifications in $tRNA^{Leu}_{UAG}$ transcript by the S-30 (Figure 2A) were expected to be derived from Trm1 activity. Trm1 enzymes can be divided into two types on the basis of their specificity for the target guanosine(s). One is a single-site-specific

Trm1, which modifies only G26 and is found in eukaryotes and archaea [57–60]. The second is a multi-site-specific Trm1, which modifies both G26 and G27 and is found in the hyperthermophilic eubacterium, *A. aeolicus* [52]. In addition to the *Ta0997* gene product (Figure 5A lane 1), we prepared two types of Trm1 enzyme from *Thermococcus kodakarensis* (Figure 5A lane 2) and *A. aeolicus* (Figure 5A lane 3) as controls. The *Ta0997* gene product methylated the tRNA^{Leu}_{UAG} transcript (data not shown) and ¹⁴C-nucleotide analysis revealed that the modified nucleotide was pm²G (Figure 5B). These results showed that the *Ta0997* gene product is the *T. acidophilum* Trm1 protein. To distinguish the site specificity, yeast tRNA^{Phe} and *A. aeolicus* tRNA^{Tyr} transcripts were prepared (Figure 5C). These tRNA transcripts were used previously to assess the site specificity of *A. aeolicus* Trm1 [52]. Yeast tRNA^{Phe} contains the sequence G26C27, whereas *A. aeolicus* tRNA^{Tyr} contains the sequence A26G27. In addition, the *A. aeolicus* tRNA^{Tyr} A26G, G27A mutant transcript has the sequence G26A27. As shown in Figure 5D, *T. acidophilum* Trm1 methylated yeast tRNA^{Phe} and *A. aeolicus* tRNA^{Tyr} A26G, G27A transcripts, which contain G26. In contrast, *T. acidophilum* Trm1 did not methylate the wild-type tRNA^{Tyr} transcript (Figure 5D center), which contains A26. Thus, these results demonstrate that *T. acidophilum* Trm1 is a single-site-specific Trm1, which methylates only G26. Similar to *T. acidophilum* Trm1, *T. kodakarensis* Trm1 methylated only the yeast tRNA^{Phe} and *A. aeolicus* tRNA^{Tyr} A26G, G27A transcripts (Figure 5E). In contrast, *A. aeolicus* Trm1 methylated all the transcripts (Figure 5F), which indicates that *A. aeolicus* Trm1 has multi-site specificity.

2.6. *T. acidophilum* Trm1 Can Modify G26 in Initiator tRNA^{Met} Transcript to m²G26 via m²G26

The G26 modification in initiator tRNA^{Met} was reported to be m²G ([25] and Table 1). Consequently, we investigated whether *T. acidophilum* Trm1 can modify G26 in the initiator tRNA^{Met} transcript to m²G. Given that the 5'-end of initiator tRNA^{Met} is an A, T7 RNA polymerase did not synthesize the transcript efficiently. Consequently, the initiator tRNA^{Met} transcript was synthesized with a 5'-leader sequence (Figure 6A) by T7 RNA polymerase (Figure 6B, lane 1) and then the 5'-leader sequence was removed with *E. coli* RNase P ([61] and Figure 6B, lane 2). The initiator tRNA^{Met} transcript was then purified by 10% polyacrylamide gel electrophoresis in the presence of 7 M urea (PAGE (7 M urea)), (Figure 6B lane 3). Trm1 from *T. acidophilum* efficiently methylated the initiator tRNA^{Met} transcript (data not shown), and the methylated nucleotide was pm²G (Figure 6C). Furthermore, the modified nucleoside analysis showed that native initiator tRNA^{Met} contained m²G (Figure 3A). Taking these results together, we conclude that initiator tRNA^{Met} from *T. acidophilum* contained the m²G26 modification in addition to m²G26 and that TrmI activity was responsible for these modifications.

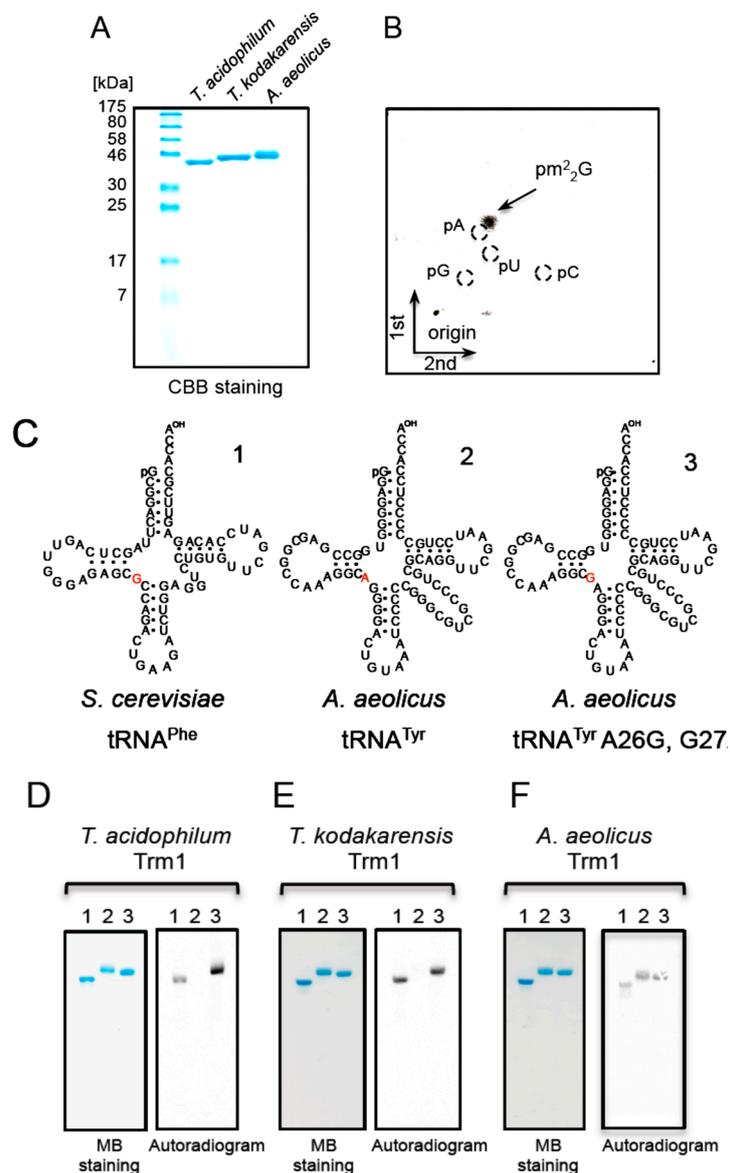


Figure 5. *T. acidophilum* Trm1 (the *Ta0997* gene product) is a single-site-specific enzyme. (A) The *T. acidophilum* *Ta0997* gene product (left), *T. kodakaraensis* Trm1 (center) and *A. aeolicus* Trm1 (right) (2 μ g each) were analyzed by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue; (B) The tRNA^{Leu}_{UAG} transcript was methylated by the *Ta0997* gene product and then the generated methylated nucleotide was analyzed by 2D-TLC; (C) The sequences of *S. cerevisiae* tRNA^{Phe}, *A. aeolicus* tRNA^{Tyr} and *A. aeolicus* tRNA^{Tyr} A26G, G27A are depicted by cloverleaf structures. These tRNA transcripts were previously used to determine the site specificity of *A. aeolicus* Trm1, which is a multi-site-specific Trm1. The nucleotides at position 26 in the tRNA transcripts are colored red. The transcript numbers (1, 2, and 3) correspond to the lane numbers in panels (D–F). (D) The tRNA transcripts (0.1 A260 units each) were incubated with the *Ta0997* gene product (*T. acidophilum* Trm1) and ¹⁴C-AdoMet at 50 °C for 5 min, separated by 10% PAGE (7 M urea), and then the gel was analyzed by autoradiography. The left and right panels show the gel stained with methylene blue and its autoradiogram, respectively. *T. kodakaraensis* (E) and *A. aeolicus* (F) Trm1 proteins were analyzed by the same method.

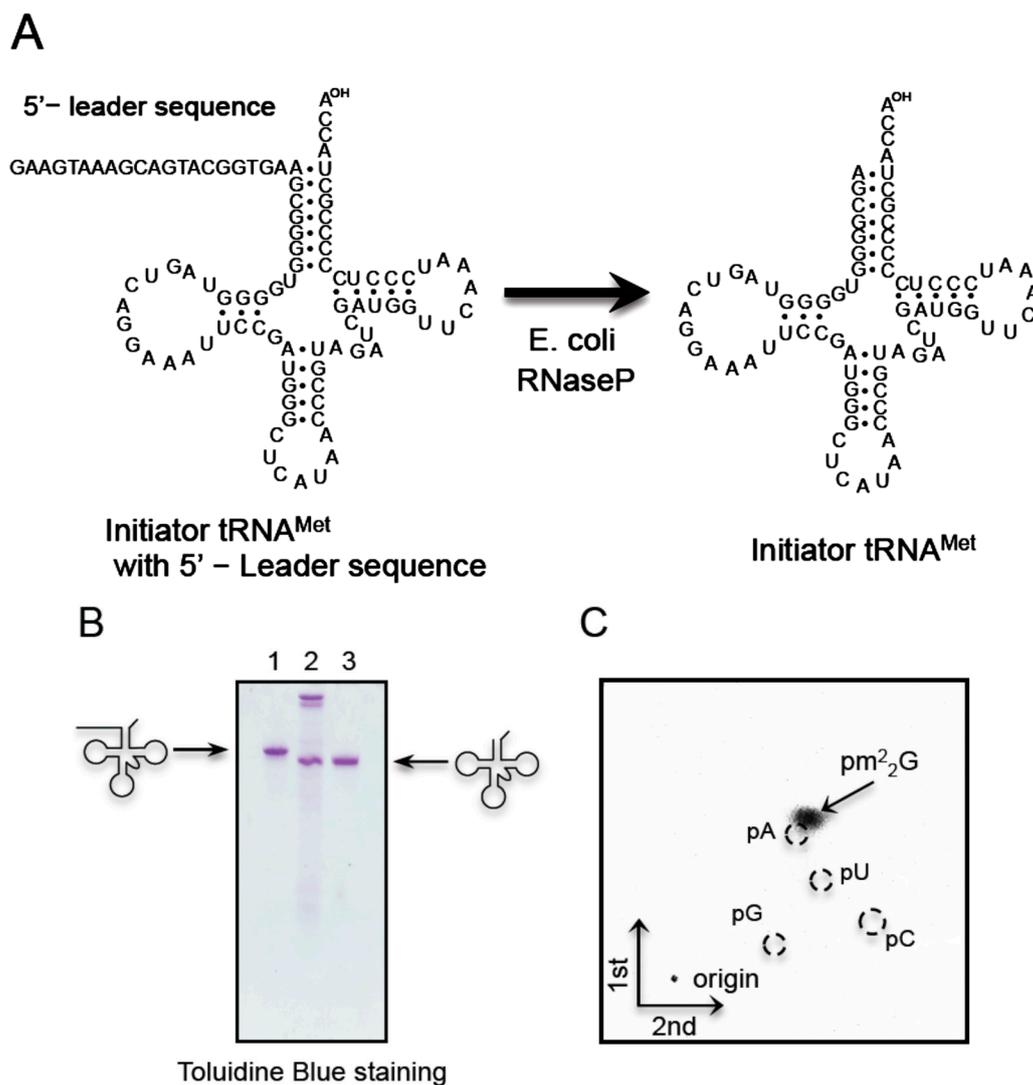


Figure 6. *T. acidophilum* Trm1 methylates the initiator tRNA^{Met} transcript. (A) Given that the 5'-end of the *T. acidophilum* initiator tRNA^{Met} is A, the transcript is not synthesized efficiently by T7 RNA polymerase. Consequently, the initiator tRNA^{Met} transcript was synthesized with a 5'-leader sequence by T7 RNA polymerase, purified by 10% PAGE (7 M urea), and then the 5'-leader sequence was removed with *E. coli* RNase P; (B) The cleavage of the 5'-leader sequence was monitored by 10% PAGE (7 M urea). The gel was stained with toluidine blue. The lanes are as follows: **left**, initiator tRNA^{Met} with the 5'-leader sequence; **center**, the reaction mixture; **right**, purified mature initiator tRNA^{Met} transcript. The M1 RNA in RNase P can be observed at the top of the gel in the center lane; (C) The methylated nucleotide was analyzed by 2D-TLC.

2.7. *T. acidophilum* Trm1 Can Methylate the Precursor of Elongator tRNA^{Met} with an Intron

The G26 modification of elongator tRNA^{Met} was uncharacterized ([24], and Table 1). Furthermore, the precursor of elongator tRNA^{Met} contains an intron at the canonical position between nucleotides 38 and 39 (Figure 1D). To verify whether Trm1 could methylate the elongator tRNA^{Met} transcript and its precursor, we analyzed the methyl group acceptance activities of these RNAs (Figure 7A). Both the

elongator tRNA^{Met} transcript and its precursor were methylated efficiently by *T. acidophilum* Trm1. The analysis of modified nucleotides by 2D-TLC revealed that methylated nucleotides were pm²G and pm²₂G (Figure 7B). The analysis of modified nucleosides revealed that native elongator tRNA^{Met} contained m²G and m²₂G (Figure 3B). Furthermore, m²G and m²₂G modifications were not reported in the published sequence of elongator tRNA^{Met} although the modification at G26 was uncharacterized [24]. Taking these results together, we conclude that the uncharacterized G26 modification in the elongator tRNA^{Met} is a mixture of m²G and m²₂G, which is formed by Trm1.

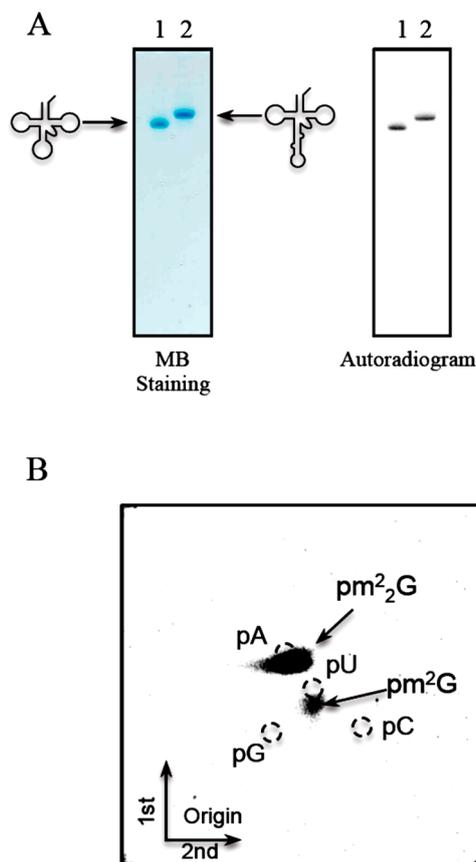


Figure 7. *T. acidophilum* Trm1 can methylate both the elongator tRNA^{Met} transcript and its precursor, which contains an intron. The mature transcript of elongator tRNA^{Met} and its precursor with an intron (Figure 1D) were prepared. (A) These transcripts were incubated with *T. acidophilum* Trm1 and ¹⁴C-AdoMet at 50 °C for 5 min, and then separated by 10% PAGE (7 M urea) (left panel). The gel was stained with methylene blue. The samples are as follows: lane 1, mature transcript of elongator tRNA^{Met}; lane 2, precursor of elongator tRNA^{Met} with an intron. The right panel shows an autoradiogram of the same gel; (B) The ¹⁴C-methylated nucleosides in the mature transcript of elongator tRNA^{Met} were analyzed by 2D-TLC.

2.8. Ta0931 Gene Product Is Trm56

To analyze whether the *Ta0931* gene product was Trm56, the recombinant protein was purified as shown in Figure 8A. The purified *Ta0931* methylated the tRNA^{Leu}_{UAG} transcript (data not shown) and the methylated nucleotide was identified as pCm (Figure 8B). The Cm modification is only found at

position 56 in native tRNA^{Leu}_{UAG} (Figure 1C). These results showed that the *Ta0931* gene product is Trm56. Neither the S-30 nor the S-100 fraction contained activity that was responsible for introducing the Cm modification into the tRNA^{Leu}_{UAG} transcript (Figure 2); however, the genome does encode Trm56. This discrepancy is addressed in the Discussion section. Finally, we investigated the influence of the presence of intron on Trm56 activity. Trm56 methylated both the elongator tRNA^{Met} transcript and its precursor (Figure 8C). However, the methyl group acceptance activity of the precursor was considerably lower than that of the mature transcript (Figure 8D). It should be mentioned that the incubation in Figure 8C was performed for 12 h to show the methylation of the precursor tRNA. These results suggest that the methylation by Trm56 occurs mainly after the removal of the intron. Although the mechanism by which Trm56 recognizes tRNA has not been reported thus far, the results of the current study suggest two possibilities. The first is that the presence of the intron results in steric hindrance that prevents Trm56 binding to the substrate tRNA. The second is that Trm56 directly recognizes the anticodon loop in the tRNA. To clarify the mechanism, further study is required.

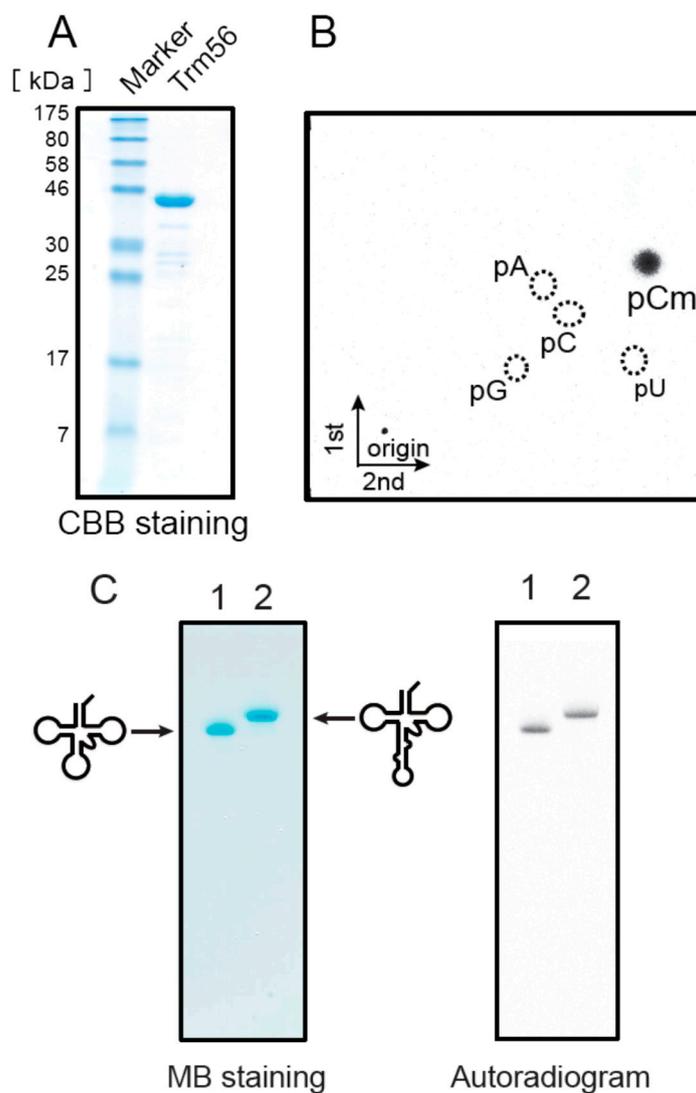


Figure 8. Cont.

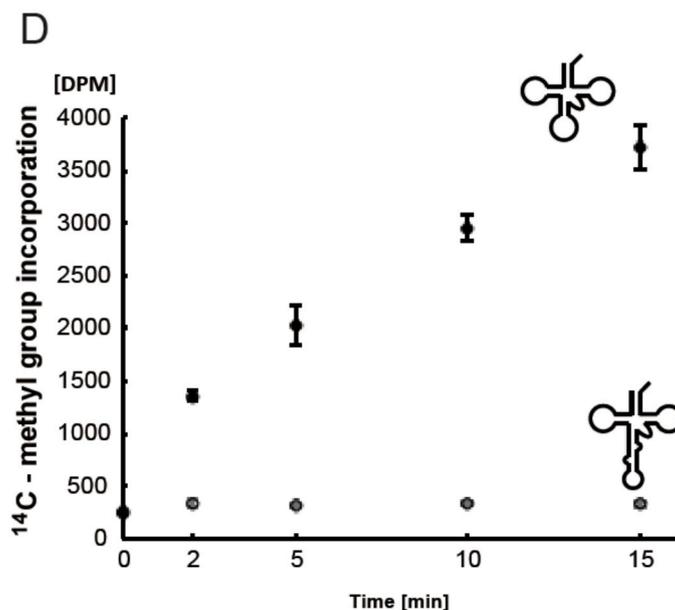


Figure 8. The *Ta0931* gene product (Trm56) can methylate both the elongator tRNA^{Met} transcript and its precursor with an intron. (A) An aliquot of 4 µg of the *Ta0931* gene product was analyzed by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue; (B) The *Ta0931* gene product and tRNA^{Leu}_{UAG} transcript were incubated at 50 °C for 1 h, and then the ¹⁴C-methylated nucleotide was analyzed by 2D-TLC; (C) The incorporation of methyl groups into the mature transcript and the precursor of elongator tRNA^{Met} were investigated. The samples were as follows: lane 1, mature transcript of elongator tRNA^{Met}; lane 2, precursor of elongator tRNA^{Met}. The gel was stained with methylene blue (**left panel**) and the autoradiogram of the same gel was taken (**right panel**); (D) The incorporation of methyl groups into the mature transcript and the precursor of elongator tRNA^{Met} were measured by the filter assay. Closed and open circles show the incorporation of ¹⁴C-methyl groups into the mature transcript and precursor of elongator tRNA^{Met}, respectively.

3. Discussion

In the current study, we investigated tRNA methyltransferase activities in crude extract from *T. acidophilum* cells, analyzed the modified nucleosides in native initiator and elongator tRNA^{Met}, and characterized three tRNA methyltransferases (Trm5, Trm1 and Trm56) by purified recombinant proteins. We utilized the sequences of three tRNAs from *T. acidophilum* (initiator tRNA^{Met}, elongator tRNA^{Met} and tRNA^{Leu}_{UAG}), which were reported previously in earlier [24,25] and our recent [28] studies, as a basis to predict the candidate genes for the enzymes responsible for the modifications. As summarized in Table 1, our experiments revealed that the genes *Ta0997*, *Ta0931*, and *Ta0836* encode Trm1, Trm56 and Trm5, respectively, from *T. acidophilum*. In archaeal tRNA modifications, there are some reports that different enzymes modify the same modification at the same position in tRNA: for example, Cm56 modification is formed by two systems, Trm56 or C/D sRNP [30]. Therefore, to understand tRNA modification systems precisely, the construction of gene disruptant mutant strains is desirable. However, there is no gene disruption method for *T. acidophilum*. Therefore, in this study, we could not utilize this approach. Consequently, the other gene product except for Trm1, Trm5 and

Trm56 may bring the same modification(s) at the same position(s) in tRNA. Unexpectedly, we could not detect Trm56 activity in the crude cell extracts (S-30 and S-100 fractions). Analysis of the *T. acidophilum* proteome revealed that various proteins form several large (more than 300 kDa) protein complexes and that some of these protein complexes appear to interact with the membrane [48]. Therefore, *T. acidophilum* Trm56 might be part of a large complex with other proteins in living cells. Similar to the Trm56 activity, we could not detect tRNA (m⁷G49) methyltransferase activity in the crude cell extract. To clarify the intracellular localization of these enzymes, further study will be required. Although the recognition of tRNA by Trm56 might be affected by the presence of other proteins in the putative complex, the purified enzyme at least can act on both the mature elongator tRNA^{Met} transcript and its precursor, which contains an intron at the canonical site. The methyl group was transferred to the mature transcript much more rapidly than to the precursor tRNA with the intron. This result suggests that the methylation by Trm56 occurs mainly after the removal of the intron. The activity of Trm5 was clearly detected in the S-100 fraction and the purified recombinant Trm5 methylated the G37 nucleotide in the tRNA^{Leu}_{UAG} transcript. Among the tRNA methyltransferases from *T. acidophilum*, only Trm5 seemed to act as a free enzyme, *i.e.*, was not included in a protein complex. It has been reported that Trm5 recognizes the tertiary interaction between the D- and T-arms [55,62]. Consequently, Trm5 might act mainly during and/or after the three-dimensional core of the tRNA has been reinforced structurally by the introduction of other modifications.

The sequence of the initiator tRNA^{Met} that is encoded in the genome of *T. acidophilum* strain HO-62 [3] differs from that reported in the earlier study [25]. The sequences of the two initiator tRNA^{Met} differ in the D-arm and at position 57: A20b and C22 are inserted in strain HO-62 and this strain also contains A57 instead of G57. These differences might be derived from the different origins of the strains: the strain HO-62 was isolated from Hakone, Japan [3]. There is another possibility as follows. The m¹I 57 modification in archaeal tRNA and the initiator tRNA^{Met} gene from *T. acidophilum* were not reported in 1982. The authors used the Kuchino's post-labelling method for tRNA sequencing [25], in which tRNA is partially cleaved by formamide and then the nucleotide at the 5'-end of each fragment is analyzed by 2D-TLC [46,63]. However, the mobility of pm¹I on 2D-TLC closely resembles that of pm²G [64]. Furthermore, in general, formamide cleavage of tRNAs from thermophiles is very difficult due to their structural rigidity. Therefore, it might be difficult technically to distinguish the pm¹I and pm²G on 2D-TLC. In the current study, we detected the m¹A (m⁶A) formation into the tRNA^{Leu}_{UAG} transcript by the S-30 fraction. However we could not identify the *trmI* gene, which encodes archaeal tRNA (m¹A57/m¹A58) methyltransferase. Consequently, we could not verify whether *T. acidophilum* TrmI can methylate the A57 in the initiator tRNA^{Met}, although our analysis of modified nucleosides revealed that m¹I is contained in the initiator tRNA^{Met}. To determine the position of m¹I modification, the RNA sequence of initiator tRNA^{Met} is required. We are now checking the plasmid vector for TrmI expression and the other gene products. From the results of the current study, we were able to add the following information with respect to initiator tRNA^{Met} from *T. acidophilum*: (1) initiator tRNA^{Met} from *T. acidophilum* strain HO-62 contains the modifications G⁺, m¹I, and m²₂G; (2) the m²₂G26 modification exists in addition to m²G26; (3) m²G26 and m²₂G26 are formed by Trm1.

In the current study, we have demonstrated that archaeal Trm1 can methylate the tRNA^{Leu}_{UAG} transcript, which has a long variable region. As far as we know, this is the first time that archaeal Trm1 has been shown to act on class II tRNAs. In the case of class I tRNAs, archaeal Trm1 was reported

to recognize the D-stem and the size of the variable region [58]. Therefore, archaeal Trm1 might be able to recognize the large variable region in the class II tRNAs. Furthermore, we showed that Trm1 efficiently methylated both the mature elongator tRNA^{Met} transcript and the precursor with an intron. These results agree well with those of a previous study [58], namely, that archaeal Trm1 does not recognize the anticodon loop. Moreover, from the results of the current study, we were able to add the following information in relation to elongator tRNA^{Met} from *T. acidophilum*: the unidentified G modification at position 26 is a mixture of m²G and m²₂G, which is formed by Trm1.

During the course of the current study, it has been reported that *Sulfolobus acidocaldarius* TrmJ is responsible for the Cm32 modification in tRNA [65]. The Cm32 modifications in initiator and elongator tRNA^{Met} are probably formed by this new enzyme as shown in Table 1.

4. Experimental Section

4.1. Materials

[Methyl-¹⁴C]-AdoMet (2.14 GBq/mmol) was purchased from PerkinElmer (Tokyo, Japan). Q-Sepharose Fast Flow, HiTrap Q-Sepharose, HiTrap SP-Sepharose, HiTrap Heparin-Sepharose and HiLoad 16/600 Superdex 200 prep grade were bought from GE Healthcare Japan (Tokyo, Japan). DNA oligomers were synthesized by Invitrogen Japan (Tokyo, Japan). Other chemical reagents were of analytical grade.

4.2. Strain, Medium, and Culture

The culture source of *T. acidophilum* HO-62 was a gift from Prof. Akihiko Yamagishi (Tokyo University of Pharmacy and Life Sciences, Hachioji, Japan). The culture was performed at 56 °C under microaerophilic conditions as described previously [3]. The culture medium contained following components (in grams per liter): yeast extract, 1.0; Casamino acid, 1.0; (NH₄)₂SO₄, 1.3; NaCl, 0.2; KH₂PO₄, 0.3; MgSO₄, 0.25; CaCl₂, 0.05; pH was adjusted to 1.8 with H₂SO₄. Cells in late log-phase were used for the experiments.

4.3. Preparation of S-30 and S-100 Fractions, and Detection of tRNA Methyltransferase Activities

Wet cells (0.3 g) were suspended in 2 mL of buffer A (50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 6 mM 2-mercaptoethanol, and 50 mM KCl). The cells were ground in a mortar with 0.15 g aluminum oxide and then the suspension was centrifuged at 8000× g for 20 min. The supernatant fraction was centrifuged further at 30,000× g for 2 h. The resultant supernatant fraction was used as the S-30 fraction. The S-100 fraction was the supernatant fraction by that was obtained after centrifugation at 100,000× g for 2 h. Transfer RNA methyltransferase activities in the S-30 and S-100 fractions were analyzed as follows: 30 µg of protein from the S-30 or S-100 fraction, 0.2 A₂₆₀ units tRNA^{Leu}_{UAG} transcript and 0.78 nmol [methyl-¹⁴C]-AdoMet were incubated in 40 µL of buffer A at 55 °C for 1 h. The RNA was extracted with phenol-chloroform and then recovered by ethanol precipitation. The RNA pellet was dissolved in 3 µL of 50 mM sodium acetate (pH 5.0), and digested with 2.5 units of nuclease P1 (Wako Pure Chemicals, Osaka, Japan). The sample was separated using 2D-TLC as described previously [64]. The

¹⁴C-methylated nucleotides were monitored with a BAS 2000 Bio-imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

4.4. Purification of Initiator and Elongator tRNA^{Met} by the Solid-Phase DNA Probe Method

Initiator and elongator tRNA^{Met} were purified by the solid-phase DNA probe method as described in our previous reports [51,52]. The sequences of the 3'-biotinylated DNA oligomers were as follows: for initiator tRNA^{Met}, 5'-ATG AGC CCA TTG GGA TTT CCT GA-biotin 3'; for elongator tRNA^{Met}, 5'-ATG AGT CCG GTG CTC CTC CAG-biotin 3'. The complementary regions are illustrated in Figure 1A,B. The isolated tRNAs were further purified by 10% PAGE (7 M urea).

4.5. Nucleoside Analysis

Nucleoside analysis was performed as described in our previous reports [50,53]. The standard marker of G⁺ was kindly provided by Prof. Takashi Yokogawa (Gifu University, Gifu, Japan).

4.6. Selection of Candidate Genes

We searched for the candidate genes in the *T. acidophilum* HO-62 genome by performing a BLAST search using the amino acid sequences of *H. volcanii* Trm1 and Trm56, and *M. jannaschii* Trm5. The identification of the other candidate genes was reported in our previous paper [28].

4.7. Cloning of the Candidate Genes, and Expression of Gene Products

4.7.1. Cloning of Ta0836 (trm5)

The *Ta0836* gene was amplified by the polymerase chain reaction (PCR) from genomic DNA from *T. acidophilum* using the following primers: Ta Trm5F, 5'-GAG ATA TAC ATA TGC CTC CAA AGA AGT TCG TTA-3'. Ta Trm5R, 5'-CTC GAA TTC GGA TCC TTA TTA CTA TGC CTT CTT AAG GGT CAT CG-3'.

4.7.2. Cloning of Ta0997 (trm1)

The *Ta0997* gene was amplified by PCR from the genomic DNA using the following primers: Ta Trm1F, 5'-GAG ATA TAC ATA TGA TAG TGA GGG AGG GTT CAG-3'; Ta Trm1R, 5'-CTC GAA TTC GGA TCC TTA TTA TGC CGG CGA TCG TCT GTG CA-3'.

4.7.3. Cloning of Ta0931 (trm56)

The *Ta0931* gene was amplified by PCR from the genomic DNA using the following primers: Ta Trm56F, 5'-GGA GAT ATA TAC ATA TGA TAA CCG TAC TGC GGA TAA ATC AC-3'; Ta Trm56R, 5'-CTC GAA TTC GGA TCC TTA TTA GCG TAT TTC ATC GAT ATC CAT ACC-3'.

4.7.4. Expression of Gene Products

The underlined regions show restriction enzyme sites (Nde I and Bam HI). The PCR products were individually inserted individually into the multiple cloning linker of expression vector pET-30a (Novagen, Cambridge, MA, USA). The gene products were expressed in the *E. coli* BL21 (DE3) Rosetta 2 strain (Novagen) in accordance with the manufacturer's instructions.

4.8. Purification of Recombinant Proteins

4.8.1. Purification of Trm5

Briefly, Trm5 was purified by heat treatment at 50 °C for 30 min, followed by successive rounds of column chromatography through HiTrap Q-Sepharose, HiTrap Heparin-Sepharose, and Toyopearl CM-650M (Tosoh, Tokyo, Japan). The final eluted sample was dialyzed against buffer B (50 mM Tris-HCl (pH 7.6), 50 mM KCl, 6 mM 2-mercaptoethanol and 5% glycerol) and concentrated with a Vivaspin 15R centrifugal filter device (Sartorius Japan, Tokyo, Japan). Glycerol was added to the sample to a final concentration of 50% v/v and the sample stored at -30 °C.

4.8.2. Purification of Trm1

Trm1 was purified as described previously [52].

4.8.3. Purification of Trm56

Briefly, Trm56 was purified by heat treatment at 50 °C for 30 min, followed by successive rounds of column chromatography through HiTrap Q-Sepharose, HiTrap Heparin-Sepharose, and HiLoad 16/600 Superdex 200 pg. The final eluted protein was dialyzed against buffer B, and concentrated with a Vivaspin 15R centrifugal filter device. Glycerol was added to the purified protein to a final concentration of 50% v/v and the samples stored at -30 °C.

4.9. Measurement of tRNA Methyltransferase Activities

The transcripts were prepared by using T7 RNA polymerase and purified by Q-Sepharose column chromatography and 10% PAGE (7 M urea). The standard assay for the purified enzymes was to measure the incorporation of ¹⁴C-methyl groups from [methyl-¹⁴C]-AdoMet into the appropriate tRNA transcript. For the reaction, 66 nM enzyme, 4.25 μM transcript, and 17.3 μM [methyl-¹⁴C]-AdoMet were incubated in 40 μL of buffer A at 50 °C for 5 min. An aliquot (35 μL) of the reaction was then used for the filter assay. To visualize the methyl-transfer reaction, we used 10% PAGE (7 M urea) and autoradiography. Briefly, tRNA (0.1 A₂₆₀ units) was incubated with 66 nM enzyme and 17.3 μM [methyl-¹⁴C]-AdoMet at 50 °C for 5 min in 40 μL of buffer A, and then loaded onto a 10% polyacrylamide gel that contained 7 M urea. The gel was stained with methylene blue or toluidine blue, and then dried. The incorporation of ¹⁴C-methyl groups into the tRNA was monitored with a Typhoon FLA 7000 laser scanner (GE Healthcare). The 2D-TLC was performed as follows. An aliquot of 1 μg of purified protein, 0.2 A₂₆₀ units of tRNA transcript and 0.69 nmol of [methyl-¹⁴C]-AdoMet were incubated in 40 μL of buffer A at 55°C for 1 h. The RNA was extracted with phenol-chloroform and then recovered by ethanol

precipitation. The RNA pellet was digested with 1.5 units of nuclease P1. The sample was separated using 2D-TLC as described previously [64]. The ^{14}C -methylated nucleotides were monitored with a Typhoon FLA 7000 laser scanner (GE Healthcare).

4.10. Preparation of *E. coli* RNase P and Removal of 5'-Leader Sequence

The plasmid vectors for the C5 protein and M1 RNA of *E. coli* RNase P were a gift from Prof. Takashi Yokogawa (Gifu University). The C5 protein was purified as described in the reference [61]. The M1 RNA was synthesized with T7 RNA polymerase. Active RNase P was generated by mixing C5 protein and M1 RNA in accordance with the method described in the reference [61]. The 5'-leader sequence of *E. coli* tRNA^{Met}f was used. The 5'-leader sequence was cleaved by RNase P in accordance with the reference [61].

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Author Contributions

Takuya Kawamura, Ryou Anraku, Chie Tomikawa and Hiroyuki Hori conceived and designed the experiments; Takuya Kawamura, Ryou Anraku, Takahiro Hasegawa, and Chie Tomikawa performed the experiments; all the authors analyzed the data; Takuya Kawamura, Chie Tomikawa and Hiroyuki Hori wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Darland, G.; Brock, T.D.; Samsonoff, W.; Conti, S.F. A thermophilic, acidophilic mycoplasma isolated from a coal refuse pile. *Science* **1970**, *170*, 1416–1418.
2. Mayberry-Carson, K.J.; Roth, I.L.; Harris, J.L.; Smith, P.F. Scanning electron microscopy of *Thermoplasma acidophilum*. *J. Bacteriol.* **1974**, *120*, 1472–1475.
3. Yasuda, M.; Oyaizu, H.; Yamagishi, A.; Oshima T. Morphological variation of new *Thermoplasma acidophilum* isolates from Japanese hot springs. *Appl. Environ. Microbiol.* **1995**, *61*, 3482–3485.
4. Shimada, H.; Nemoto, N.; Shida Y.; Oshima, T.; Yamagishi, A. Complete polar lipid composition of *Thermoplasma acidophilum* HO-62 determined by high-performance liquid chromatography with evaporative light-scattering detection. *J. Bacteriol.* **2002**, *184*, 556–563.

5. Posner, M.G.; Upadhyay, A.; Bagby, S.; Hough, D.W.; Danson, M.J. A unique lipoylation system in the Archaea lipoylation in *Thermoplasmata acidophilum* requires two proteins. *FEBS J.* **2009**, *276*, 4012–4022.
6. Xu, Q.; Eguchi, T.; Mathews, I.I.; Rife, C.L.; Chiu, H.J.; Farr, C.L.; Feuerhelm, J.; Jaroszewski, L.; Klock, H.E.; Knuth, M.W.; *et al.* Insights into substrate specificity of geranylgeranyl reductases revealed by the structure of digeranylgeranyl glycerophospholipid reductase, an essential enzyme in the biosynthesis of archaeal membrane lipids. *J. Mol. Biol.* **2010**, *404*, 403–417.
7. Vinogradov, E.; Deschatelets, L.; Lamoureux, M.; Patel, G.B.; Tremblay, T.L.; Robotham, A.; Goneau, M.F.; Cummings-Lorbetskie, C.; Watson, D.C.; Brisson, J.R.; *et al.* Cell surface glycoproteins from *Thermoplasma acidophilum* are modified with an *N*-linked glycan containing 6-*C*-sulfofucose. *Glycobiology* **2012**, *22*, 1256–1267.
8. Kloda, A.; Martinac, B. Mechanosensitive channel of *Thermoplasma*, the cell wall-less archaea: Cloning and molecular characterization. *Cell Biochem. Biophys.* **2001**, *34*, 321–347.
9. Stein, D.B.; Sercy, D.G. Physiologically important stabilization of DNA by a prokaryotic histone-like protein. *Science* **1978**, *202*, 219–221.
10. DeLange, R.J. A histone-like protein (HTa) from *Thermoplasma acidophilum*. *J. Biol. Chem.* **1981**, *256*, 900–904.
11. Haugland, G.T.; Shin, J.H.; Birkeland, N.K.; Kelman, Z. Stimulation of MCM helicase activity by a Cdc6 protein in the archaeon *Thermoplasma acidophilum*. *Nucleic Acids Res.* **2006**, *34*, 6337–6344.
12. Ogino, H.; Ishino, S.; Mayanagi, K.; Haugland, G.T.; Birkeland, N.K.; Yamagishi, A.; Ishino, Y. The GINS complex from the thermophilic archaeon, *Thermoplasma acidophilum* may function as a homotetramer in DNA replication. *Extremophiles* **2011**, *15*, 529–539.
13. Roth, H.M.; Tessmer, I.; van Houten, B.; Kisker, C. Bax1 is a novel endonuclease: Implications for archaeal nucleotide excision repair. *J. Biol. Chem.* **2009**, *284*, 32272–32278.
14. Moen, M.N.; Knævelsrud, I.; Haugland, G.T.; Grøsvik, K.; Birkeland, N.K.; Klungland, A.; Bjelland, S. Uracil-DNA glycosylase of *Thermoplasma acidophilum* directs long-patch base excision repair, which is promoted by deoxynucleoside triphosphates and ATP/ADP, into short-patch repair. *J. Bacteriol.* **2011**, *193*, 4495–4508.
15. Kuper, J.; Wolski, S.C.; Michels, G.; Kisker, C. Functional and structural studies of the nucleotide excision repair helicase XPD suggest a polarity for DNA translocation. *EMBO J.* **2012**, *31*, 494–502.
16. Buechner, C.N.; Heil, K.; Michels, G.; Carell, T.; Kisker, C.; Tessmer, I. Strand-specific recognition of DNA damages by XPD provides insights into nucleotide excision repair substrate versatility. *J. Biol. Chem.* **2014**, *289*, 3613–3624.
17. Sun, N.; Pan, C.; Nickell, S.; Mann, M.; Baumeister, W.; Nagy, I. Quantitative proteome and transcriptome analysis of the archaeon *Thermoplasma acidophilum* cultured under aerobic and anaerobic conditions. *J. Proteome Res.* **2010**, *9*, 4839–4850.
18. Kim, S.M.; Paek, K.H.; Lee, S.B. Characterization of NADP⁺-specific *L*-rhamnose dehydrogenase from the thermoacidophilic Archaeon *Thermoplasma acidophilum*. *Extremophiles* **2012**, *16*, 447–454.
19. Ruepp, A.; Graml, W.; Santos-Martinez, M.L.; Koretke, K.K.; Volker, C.; Mewes, H.W.; Frishman, D.; Stocker, S.; Lupas, A.N.; Baumeister, W. The genome sequence of the thermoacidophilic scavenger *Thermoplasma acidophilum*. *Nature* **2000**, *407*, 508–813.
20. Brandstetter, H.; Kim, J.-S.; Groll, M.; Huber, R. Crystal structure of the tricorn protease reveals a protein disassembly line. *Nature* **2001**, *414*, 466–470.

21. Steinbacher, S.; Ditzel, L. Review: Nucleotide binding to the *Thermoplasma* thermosome: Implications for the functional cycle of group II chaperonins. *J. Struct. Biol.* **2001**, *135*, 147–156.
22. Noi, K.; Hirai, H.; Hongo, K.; Mizobata, T.; Kawata, Y. Implications for the functional cycle of group II chaperonins from *Thermoplasma acidophilum*. *Biochemistry* **2009**, *48*, 9405–9415.
23. Ruschak, A.M.; Religa, T.L.; Breuer, S.; Witt, S.; Kay, L.E. The proteasome antechamber maintains substrates in an unfolded state. *Nature* **2010**, *467*, 868–873.
24. Kilpatrick, M.W.; Walker, R.T. The nucleotide sequence of the tRNA_{M^{Met}} from the archaeobacterium *Thermoplasma acidophilum*. *Nucleic Acids Res.* **1981**, *9*, 4387–4390.
25. Kuchino, Y.; Ihara, M.; Yabusaki, Y.; Nishimura, S. Initiator tRNAs from archaeobacteria show common unique sequence characteristics. *Nature* **1982**, *298*, 684–685.
26. Walker, R.T. Mycoplasma evolution: A review of the use of ribosomal and transfer RNA nucleotide sequences in the determination of phylogenetic relationships. *Yale J. Biol. Med.* **1983**, *56*, 367–372.
27. Edmonds, C.G.; Crain, P.F.; Gupta, R.; Hashizume, T.; Hocart, C.H.; Kowalak, J.A.; Pomerantz, S.C.; Stetter, K.O.; McCloskey, J.A. Posttranscriptional modification of tRNA in thermophilic archaea (Archaeobacteria). *J. Bacteriol.* **1991**, *173*, 3138–3148.
28. Tomikawa, C.; Ohira, T.; Inoue, Y.; Kawamura, T.; Yamagishi, A.; Suzuki, T.; Hori, H. Distinct tRNA modifications in the thermo-acidophilic archaeon, *Thermoplasma acidophilum*. *FEBS Lett.* **2013**, *587*, 3575–3580.
29. Gregson, J.M.; Crain, P.F.; Edmonds, C.G.; Gupta, R.; Hashizume, T.; Phillipson, D.W.; McCloskey, J.A. Structure of the archaeal transfer RNA nucleoside G*-15 (2-amino-4,7-dihydro-4-oxo-7-beta-D-ribofuranosyl-1H-pyrrolo[2,3-d]pyrimidine-5-carboximidamide (archaeosine)). *J. Biol. Chem.* **1993**, *268*, 10076–10086.
30. Renalier, M.H.; Joseph, N.; Gaspin, C.; Thebault, P.; Mougin, A. The Cm56 tRNA modification in archaea is catalyzed either by a specific 2'-O-methylase, or a C/D sRNP. *RNA* **2005**, *11*, 1051–1063.
31. Jühling, F.; Mörl, M.; Hartmann, R.K.; Sprinzl, M.; Stadler, P.F.; Pütz, J. tRNAdb 2009: Compilation of tRNA sequences and tRNA genes. *Nucleic Acids Res.* **2009**, *37*, D159–D162.
32. Machnicka, M.A.; Milanowska, K.; Osman Oglou, O.; Purta, E.; Kurkowska, M.; Olchowik, A.; Januszewski, W.; Kalinowski, S.; Dunin-Horkawicz, S.; Rother, K.M.; *et al.* MODOMICS: A database of RNA modification pathways—2013 update. *Nucleic Acids Res.* **2013**, *41*, D262–D267.
33. De Bie, L.G.; Roovers, M.; Oudjama, Y.; Wattiez, R.; Tricot, C.; Stalon, V.; Droogmans, L.; Bujnicki, J.M. The *yggH* gene of *Escherichia coli* encodes a tRNA (m⁷G46) methyltransferase. *J. Bacteriol.* **2003**, *185*, 3238–3243.
34. Okamoto, H.; Watanabe, K.; Ikeuchi, Y.; Suzuki, T.; Endo, Y.; Hori, H. Substrate tRNA recognition mechanism of tRNA (m⁷G46) methyltransferase from *Aquifex aeolicus*. *J. Biol. Chem.* **2004**, *279*, 49151–49159.
35. Alexandrov, A.; Martzen, M.R.; Phizicky, E.M. Two proteins that form a complex are required for 7-methylguanosine modification of yeast tRNA. *RNA* **2002**, *8*, 1253–1266.
36. Kambampati, R.; Lauhon, C.T. Evidence for the transfer of sulfane sulfur from IscS to ThiI during the in vitro biosynthesis of 4-thiouridine in *Escherichia coli* tRNA. *J. Biol. Chem.* **2000**, *275*, 10727–10730.
37. Lauhon, C.T.; Kambampati, R. The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. *J. Biol. Chem.* **2000**, *275*, 20096–20103.

38. Liu, Y.; Zhu, X.; Nakamura, A.; Orlando, R.; Söll, D.; Whitman, W.B. Biosynthesis of 4-thiouridine in tRNA in the methanogenic archaeon *Methanococcus maripaludis*. *J. Biol. Chem.* **2012**, *287*, 36683–36692.
39. Miranda, H.V.; Nembhard, N.; Su, D.; Hepowit, N.; Krause, D.J.; Pritz, J.R.; Phillips, C.; Söll, D.; Maupin-Furlow, J.A. E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4417–4422.
40. Phillips, G.; Swairjo, M.A.; Gaston, K.W.; Bailly, M.; Limbach, P.A.; Iwata-Reuyl, D.; de Crécy-Lagard, V. Diversity of archaeosine synthesis in Crenarchaeota. *ACS Chem. Biol.* **2012**, *7*, 300–305.
41. Keith, G.; Desgrès, J.; Pochart, P.; Heyman, T.; Kuo, K.C.; Gehrke, C.W. Eukaryotic tRNAs(Pro): Primary structure of the anticodon loop; presence of 5-carbamoylmethyluridine or inosine as the first nucleoside of the anticodon. *Biochim. Biophys. Acta* **1990**, *1049*, 255–260.
42. Chen, C.; Huang, B.; Anderson, J.T.; Byström, A.S. Unexpected accumulation of mcm⁵U and mcm⁵s²U in a *trm9* mutant suggests an additional step in the synthesis of mcm⁵U and mcm⁵s²U. *PLoS One* **2011**, *6*, e20783.
43. Grosjean, H.; Constantinesco, F.; Foiret, D.; Benachenhou, N. A novel enzymatic pathway leading to 1-methylinosine modification in *Haloferax volcanii* tRNA. *Nucleic Acids Res.* **1995**, *23*, 4312–4319.
44. Roovers, M.; Wouters, J.; Bujnicki, J.M.; Tricot, C.; Stalon, V.; Grosjean, H.; Droogmans, L. A primordial RNA modification enzyme: the case of tRNA (m¹A) methyltransferase. *Nucleic Acids Res.* **2004**, *32*, 465–476.
45. Hamdane, D.; Guelorget, A.; Guérineau, V.; Golinelli-Pimpaneau, B. Dynamics of RNA modification by a multi-site-specific tRNA methyltransferase. *Nucleic Acids Res.* **2015**, *42*, 11697–11706.
46. Kuchino, Y.; Kato, M.; Sugisaki, H.; Nishimura, S. Nucleotide sequence of starfish initiator tRNA. *Nucleic Acids Res.* **1979**, *6*, 3459–3469.
47. Constantinesco, F.; Motorin, Y.; Grosjean, H. Transfer RNA modification enzymes from *Pyrococcus furiosus*: detection of the enzymatic activities *in vitro*. *Nucleic Acids Res.* **1999**, *27*, 1308–1315.
48. Sun, N.; Tamura, N.; Tamura, T.; Knispel, R.W.; Hrabe, T.; Kofler, C.; Nickell, S.; Nagy, I. Size distribution of native cytosolic proteins of *Thermoplasma acidophilum*. *Proteomics* **2009**, *9*, 3783–3786.
49. Droogmans, L.; Roovers, M.; Bujnicki, J.M.; Tricot, C.; Hartsch, T.; Stalon, V.; Grosjean, H. Cloning and characterization of tRNA (m¹A58) methyltransferase (TrmI) from *Thermus thermophilus* HB27, a protein required for cell growth at extreme temperatures. *Nucleic Acids Res.* **2003**, *31*, 2148–2156.
50. Tomikawa, C.; Yokogawa, T.; Kanai, T.; Hori, H. N⁷-Methylguanine at position 46 (m⁷G46) in tRNA from *Thermus thermophilus* is required for cell viability through a tRNA modification network. *Nucleic Acids Res.* **2010**, *38*, 942–957.
51. Yokogawa, T.; Kitamura, Y.; Nakamura, D.; Ohno, S.; Nishikawa, K. Optimization of the hybridization-based method for purification of thermostable tRNAs in the presence of tetraalkylammonium salts. *Nucleic Acids Res.* **2010**, *38*, doi:10.1093/nar/gkp1182.

52. Awai, T.; Kimura, S.; Tomikawa, C.; Ochi, A.; Ihsanawati; Bessho, Y.; Yokoyama, S.; Ohno, S.; Nishikawa, K.; Yokogawa, T.; Suzuki, T.; Hori, H. *Aquifex aeolicus* tRNA (N^2,N^2 -guanine)-dimethyltransferase (Trm1) catalyzes transfer of methyl groups not only to guanine 26 but also to guanine 27 in tRNA. *J. Biol. Chem.* **2009**, *284*, 20467–20478.
53. Ishida, K.; Kunibayashi, T.; Tomikawa, C.; Ochi, A.; Kanai, T.; Hirata, A.; Iwashita, C.; Hori, H. Pseudouridine at position 55 in tRNA controls the contents of other modified nucleotides for low-temperature adaptation in the extreme-thermophilic eubacterium *Thermus thermophilus*. *Nucleic Acids Res.* **2011**, *39*, 2304–2318.
54. Hirata, A.; Kitajima, T.; Hori, H. Cleavage of intron from the standard or non-standard position of the precursor tRNA by the splicing endonuclease of *Aeropyrum pernix*, a hyper-thermophilic Crenarchaeon, involves a novel RNA recognition site in the Crenarchaea specific loop. *Nucleic Acids Res.* **2011**, *39*, 9376–9389.
55. Christian, T.; Evilia, C.; Williams, S.; Hou, Y.M. Distinct origins of tRNA(m^1G37) methyltransferase. *J. Mol. Biol.* **2004**, *339*, 707–719.
56. Goto-Ito S.; Ito T.; Ishii R.; Muto Y.; Bessho Y.; Yokoyama S. Crystal structure of archaeal tRNA($m(1)G37$)methyltransferase aTrm5. *Proteins* **2008**, *72*, 1274–1289.
57. Reinhart, M.P.; Lewis, J.M.; Leboy, P.S. A single tRNA (guanine)-methyltransferase from *Tetrahymena* with both mono- and di-methylating activity. *Nucleic Acids Res.* **1986**, *14*, 1131–1148.
58. Constantinesco, F.; Motorin, Y.; Grosjean, H. Characterisation and enzymatic properties of tRNA(guanine 26, N^2,N^2)-dimethyltransferase (Trm1p) from *Pyrococcus furiosus*. *J. Mol. Biol.* **1999**, *291*, 375–392.
59. Phillips, J.H.; Kjellin-Straby, K. Studies on microbial ribonucleic acid. IV. Two mutants of *Saccharomyces cerevisiae* lacking N^2 -dimethylguanine in soluble ribonucleic acid. *J. Mol. Biol.* **1967**, *26*, 509–518.
60. Ellis, S.R.; Morales, M.J.; Li, J.M.; Hopper, A.K.; Martin, N.C. Isolation and characterization of the TRM1 locus, a gene essential for the N^2,N^2 -dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1986**, *261*, 9703–9709.
61. Fukunaga J.; Gouda M.; Umeda K.; Ohno S.; Yokogawa T.; Nishikawa K. Use of RNase P for efficient preparation of yeast tRNA^{Tyr} transcript and its mutants. *J. Biochem.* **2006**, *139*, 123–127.
62. Goto-Ito, S.; Ito, T.; Kuratani, M.; Bessho, Y.; Yokoyama, S. Tertiary structure checkpoint at anticodon loop modification in tRNA functional maturation. *Nat. Struct. Mol. Biol.* **2009**, *16*, 1109–1115.
63. Stanley, J.; Vassilenko, S. A different approach to RNA sequencing. *Nature* **1978**, *274*, 87–89.
64. Grosjean, H.; Droogmans, L.; Roovers, M.; Keith, G. Detection of enzymatic activity of transfer RNA modification enzymes using radiolabeled tRNA substrates. *Methods Enzymol.* **2007**, *425*, 55–101.
65. Somme, J.; van Laer, B.; Roovers, M.; Steyaert, J.; Versées, W.; Droogmans, L. Characterization of two homologous 2'-O-methyltransferases showing different specificities for their tRNA substrates. *RNA* **2014**, *20*, 1257–1271.