



Review

Transfer RNA Modification Enzymes from Thermophiles and Their Modified Nucleosides in tRNA

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Abstract: To date, numerous modified nucleosides in tRNA as well as tRNA modification enzymes have been identified not only in thermophiles but also in mesophiles. Because most modified nucleosides in tRNA from thermophiles are common to those in tRNA from mesophiles, they are considered to work essentially in steps of protein synthesis at high temperatures. At high temperatures, the structure of unmodified tRNA will be disrupted. Therefore, thermophiles must possess strategies to stabilize tRNA structures. To this end, several thermophile-specific modified nucleosides in tRNA have been identified. Other factors such as RNA-binding proteins and polyamines contribute to the stability of tRNA at high temperatures. *Thermus thermophilus*, which is an extreme-thermophilic eubacterium, can adapt its protein synthesis system in response to temperature changes via the network of modified nucleosides in tRNA and tRNA modification enzymes. Notably, tRNA modification enzymes from thermophiles are very stable. Therefore, they have been utilized for biochemical and structural studies. In the future, thermostable tRNA modification enzymes may be useful as biotechnology tools and may be utilized for medical science.

Keywords: archaea; methylation; pseudouridine; RNA modification; tRNA methyltransferase; tRNA modification

1. Introduction

Transfer RNA is an adaptor molecule required for the conversion of genetic information encoded by nucleic acids into amino acid sequences of proteins [1,2]. Figure 1A shows typically conserved nucleosides in a tRNA molecule, which is represented as a cloverleaf structure (herein, the nucleotide positions in tRNA are numbered, according to Sprinzl et al. [3]). These conserved nucleotides are important for tRNA folding and for stabilization of the L-shaped tRNA structure (Figure 1B) [4–6]. In addition to the standard nucleosides, numerous modified nucleosides in tRNA (for structures, see the MODOMICS and tRNAmoviz databases: <http://modomics.genesilico.pl/>; <http://genesilico.pl/trnamoviz> [7]) have been discovered in both thermophilic and mesophilic tRNAs [7,8] (see Supplementary Table S1 for abbreviations of modified nucleosides).

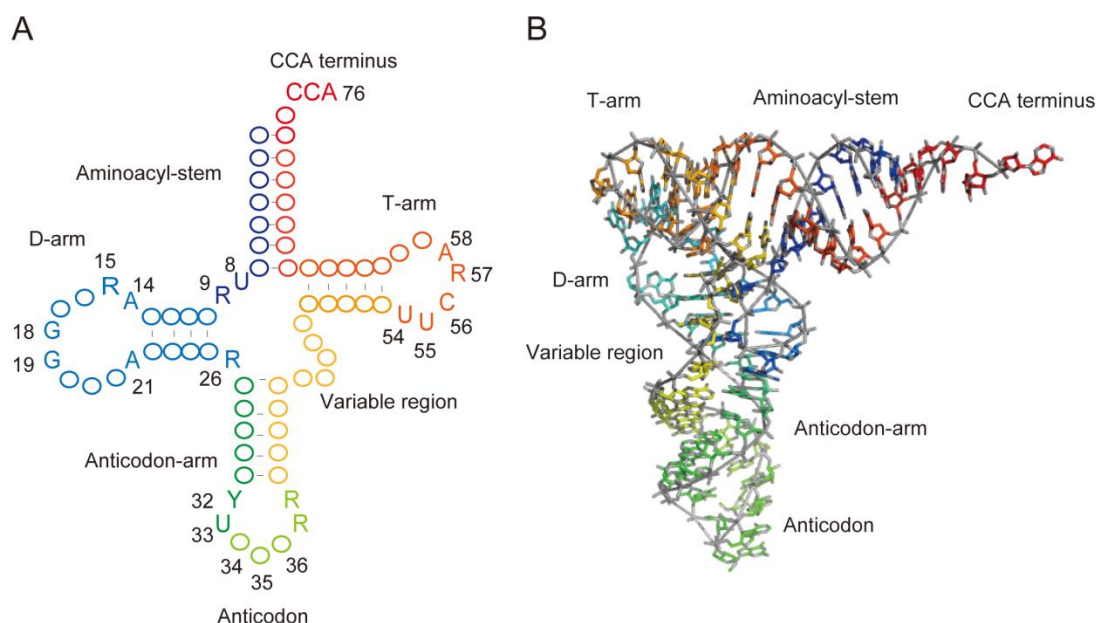


Figure 1. The structure of tRNA. **(A)** Representation of secondary structure of tRNA in a cloverleaf structure. This figure shows tRNA with a short variable region. Conserved nucleosides are shown with position numbers. Abbreviations: R, purine. Y, pyrimidine. **(B)** The L-shaped structure of *Saccharomyces cerevisiae* tRNA^{Phe}. The colors of nucleosides correspond to those in **(A)**.

A comprehensive review of the modified nucleosides in tRNA from thermophiles and their positions, distribution, predicted (or confirmed) tRNA modification enzymes and structural effects (Table 1) [9–264], which suggests that the majority of modified nucleosides in tRNA from thermophiles are common to those in tRNA from mesophiles. The functions of modified nucleosides in tRNAs have been gradually elucidated by biochemical and structural studies, physicochemical measurements, and analyses of gene disruption strains. The modified nucleosides primarily function in protein synthesis (e.g., stabilization of tRNA structure [88,265–267], correct folding of tRNA [88,265–267], reinforcement, restriction, and/or alteration of codon-anticodon interaction [108,109,114–117,120,124,268–270], recognition by aminoacyl-tRNA synthetases [109,116,117,271], recognition by translation factors [272], and prevention of the frameshift error [122,123,157,158] among others). In short, living organisms cannot synthesize proteins correctly or efficiently without modifications in tRNA.

For some organisms, modifications in tRNA have not been confirmed, but the tRNA modification enzymes have been studied. For example, although no tRNA sequences from *Thermotoga maritima* have been reported, the properties of several tRNA modification enzymes of this hyper-thermophilic eubacterium have been documented and, thus, the nucleoside modifications are predicted. Although many of the functions and biosynthesis pathways of modified nucleosides in tRNA from thermophiles have not yet been investigated, most of them are considered to be basically common to those from mesophiles. However, thermophiles live in extreme environments (e.g., high temperature, anaerobic conditions, extreme pH, and high pressure). Therefore, it is possible that tRNA modifications observed in thermophiles may have novel functions. Furthermore, in some cases, the biosynthesis pathways of some modifications may differ between thermophiles and mesophiles. Moreover, in eukaryotes, tRNA modifications are related to higher biological processes such as cellular transport of tRNA [273–278], RNA quality control [274,279–281], infection [282–286], and the immune response [287–290]. As yet, modified nucleosides in tRNA from thermophilic eukaryotes have not been investigated, but it is possible that a relationship between modified nucleosides in tRNA and these biological phenomena may also be discovered in thermophilic eukaryotes.

In this review, we focus on the modified nucleosides and tRNA modification enzymes from thermophiles including the difficulties in sequencing the rigid and stable tRNAs from thermophiles.

Since the tRNA modifications in moderate thermophiles are essentially similar to those in mesophiles, we describe them separately from extreme-thermophiles and hyper-thermophiles. We focus on the strategies for tRNA stabilization of extreme hyperthermophiles. Furthermore, we describe the potential effects of these modifications during oxidative and other environmental stresses at high temperatures. Lastly, we describe biotechnological and therapeutic uses for tRNA modification enzymes. To avoid overlap with previous publications, we intentionally refer to representative articles and reviews of modified nucleosides in tRNA and tRNA modification enzymes from mesophiles (main text and Table 1) to aid understanding by the readers. For example, tRNA modifications in archaea including mesophiles have been extensively covered [48,87,291–294] and pseudouridine modifications and methylated nucleosides in tRNA are reviewed elsewhere [87,203,295,296]. Furthermore, the stability of nucleic acids at high temperatures has been reviewed [297]. Other useful publications are pointed out in the appropriate sections throughout the review.

Table 1. Modified nucleosides in tRNA from thermophiles.

Modified Nucleoside and Position	Distrib.	Modification Enzyme	Predicted Functions and Additional Information	References
Am ₆	A	Unknown	Stabilization of aminoacyl-stem Enzymatic activity for Am ₆ formation has been detected in the cell extract of <i>Pyrococcus furiosus</i>	[9]
m ² G ₆	B/A	TrmN/Trm14	Stabilization of aminoacyl-stem	[10–15]
U ₈	A	CDAT8	Increasing G-C content in tRNA genes In <i>Methanopyrus kandleri</i> , U ₈ in several tRNA is produced from C ₈ by the deamination [16] In <i>Methanopyrus kandleri</i> , numerous nucleosides in RNA may be 2-O-methylated (see main text) [17]	[16,17]
s ⁴ U ₈	B/A	ThiI + IscS/ThiI	UV resistance in <i>E. coli</i> and <i>Salmonella typhimurium</i> (see main text) Stabilization of D-arm structure in <i>E. coli</i> (see main text)	[10,11,18–35]
s ⁴ U ₈ and s ⁴ U ₉	A	ThiI + α?	UV resistance Stabilization of D-arm structure (see main text) Sulfur-containing modifications in tRNA are reviewed in Reference [35].	[36]
m ¹ A ₉	A	Archaeal Trm10	Stabilization of the D-arm structure Prevention of formation of a Watson–Crick base pair Correct folding of the D-arm region	[37,38]
m ¹ G ₉ and m ¹ A ₉	A	archaeal Trm10	Stabilization of D-arm structure Prevention of formation of a Watson–Crick base pair Correct folding of D-arm region <i>Thermococcus kodakarensis</i> Trm10 forms m ¹ G ₉ and m ¹ A ₉ , whereas <i>Sulfolobus acidocaldarius</i> Trm10 forms only m ¹ A ₉	[37,39]
(m ² G ₁₀ and) m ² ₂ G ₁₀	A	archaeal Trm11 (Trm-G ₁₀ ; Trm-m ² ₂ G ₁₀ enzyme)	Prevention of formation of a Watson-Crick base pair Correct folding of tRNA in <i>Pyrococcus furiosus</i> Correct folding of the D-arm region	[40–43]
Ψ ₁₃	B/A	TruD/TruD or archaeal Pus7	Stabilization of D-stem structure Archaeal Pus7 generally catalyzes formation of Ψ ₃₅ in tRNA ^{Tyr} , but <i>Sulfolobus solfaraticus</i> Pus7 has weak Ψ ₁₃ formation activity [46]	[23,44–46]
G ⁺ ₁₃	A	ArcTGT + ArcS?	Stabilization of the D-arm structure <i>Thermoplasma acidophilum</i> tRNA ^{Leu} exceptionally possesses a G ⁺ ₁₃ modification and <i>T. acidophilum</i> ArcTGT acts on positions 13 and 15 in this tRNA [47]	[36,47]

Table 1. Cont.

Modified Nucleoside and Position	Distrib.	Modification Enzyme	Predicted Functions and Additional Information	References
G ⁺ ₁₅	A	ArcTGT + ArcS or QueF-like protein	Stabilization of interaction between the D-arm and the variable region Several archaea possess a split-type ArcTGT [60,61] Several species in Crenarchaeota possess a QueF-like protein instead of ArcS [60,62,63] G ⁺ is not found in nucleosides from a <i>Stetteria hydrogenophila</i> tRNA mixture [56]	[25,36,47–63]
D ₁₇	B	Dus family protein?	Maintenance of D-loop flexibility D ₁₇ and D ₂₀ modifications have been reported in <i>Geobacillus stearothermophilus</i> tRNA. However, D ₁₇ and D ₂₀ are formed by DusB and DusA, respectively, in <i>Escherichia coli</i> [65,66] and the <i>G. stearothermophilus</i> genome possesses only one <i>dus</i> -like gene. This is also observed in <i>Bacillus subtilis</i> , which is a mesophilic eubacterium.	[18,19,64–66]
Gm ₁₈	B	TrmH	Stabilization of the D-arm and the T-arm interaction. TrmH from thermophiles possess relative broad substrate tRNA specificities as compared with TrmH from <i>E. coli</i> . The substrate tRNA specificities of TrmH enzymes differ among thermophiles. TrmH from <i>Thermus thermophilus</i> can methylate all tRNA species.	[10,11,20,21,23,24,30,67–81]
D ₂₀	B	Dus family protein	Stabilization of local structure of D-loop in <i>E. coli</i> ? In <i>A. aeolicus</i> , the nucleosides at positions 20 and 20a in tRNA ^{Cys} are D ₂₀ and U _{20a} , respectively. Therefore, Dus from <i>A. aeolicus</i> may act only on U ₂₀ in tRNA.	[24,33,65,66,82]
D ₂₀ and D _{20a}	B	DusA	Stabilization of local structure of the D-loop The melting temperature of a tRNA mixture from the <i>E. coli dusA</i> gene disruptant strain is lower than that from the wild-type strain [33]. Therefore, D ₂₀ and D _{20a} modifications may contribute to stabilize local structure of the D-loop. <i>Thermus thermophilus</i> Dus was recently confirmed as a member of the DusA family [65,66,84,85].	[21–23,33,65–67,83–85]
m ¹ A ₂₂	B	TrmK	Prevention of formation of a Watson–Crick base pair	[18,20,86]
Ψ ₂₂	A	Unknown	The Ψ ₁₃ -Ψ ₂₂ base pair may stabilize D-arm structure [88]	[87,88]

Table 1. Cont.

Modified Nucleoside and Position	Distrib.	Modification Enzyme	Predicted Functions and Additional Information	References
m^2G_{26} and $m^2_2G_{26}$	A	Trm1	Stabilization of three-dimensional core structure Correct folding of tRNA Recently, it has been reported that $m^2_2G_{26}$ modification is required for correct folding of precursor tRNA ^{Ser} from <i>Schizosaccharomyces pombe</i> [94]. Therefore, a similar phenomenon may take place in thermophiles.	[9,25,44,89–94]
m^2G_{26} , $m^2_2G_{26}$, m^2G_{27} and $m^2_2G_{27}$	B	Trm1	Stabilization of three-dimensional core structure in <i>A. aeolicus</i> . In the case of m^2G_{27} and $m^2_2G_{27}$, stabilization of aminoacyl-stem	[24,95]
$m^2_2Gm_{26}$	A	Trm1 + unknown MT	Stabilization of three-dimensional core structure The presence of m^2_2Gm has been confirmed in nucleosides of a tRNA mixture from several thermophilic archaea [56,97–100]. Although the nucleoside at position 26 in <i>S. acidocaldarius</i> tRNA ^{Met₁} was originally reported as an unidentified G modification [44], it was recently described as m^2_2Gm [96]. The MT for 2'-O-methylation is unknown.	[44,96]
Cm_{32}	A	archaeal TrmJ	Stabilization of anticodon-loop	[96]
Cm_{32} and Nm_{32}	B	TrmJ	Stabilization of anticodon-loop TrmJ from <i>E. coli</i> does not recognize the base at position 32 [96,102]. Um_{32} and Am_{32} have not been reported in tRNAs from thermophilic eubacteria.	[96,101,102]
I_{34}	B	TadA	Alteration of codon–anticodon interaction A-to-I editing in tRNA is reviewed in Reference [107]	[103–107]
k^2C_{34}	B	TilS	Alteration of codon–anticodon interaction (<i>E. coli</i> and <i>B. subtilis</i>) Change of recognition by aminoacyl-tRNA synthetase (<i>E. coli</i> and <i>B. subtilis</i>) Decoding of AUA codons by k^2C_{34} and agm^2C_{34} modifications is reviewed in References [114,115].	[108–113]
agm^2C_{34}	A	TiaS	Alteration of codon–anticodon interaction (<i>Arhaeoglobus fulgidus</i> and <i>Haloarcula marismortui</i>) Change of recognition by aminoacyl-tRNA synthetase (<i>Arhaeoglobus fulgidus</i> and <i>Haloarcula marismortui</i>) Decoding of AUA codons by k^2C_{34} and agm^2C_{34} modifications is reviewed in References [114,115].	[114–120]

Table 1. Cont.

Modified Nucleoside and Position	Distrib.	Modification Enzyme	Predicted Functions and Additional Information	References
xm ⁵ U ₃₄ derivatives	B/A	MnmE + MnmG + MnmC (for mnm ⁵ U ₃₄ in eubacteria)/Elp3? + α (for cm ⁵ U ₃₄ in archaea) IscS + TusA + TusBCD + TusE + mnmA (for 2-thiolation in <i>E. coli</i>) or YrvO + mnmA (for 2-thiolation in <i>B. subtilis</i>) SAMP2 + UbaA + NcsA (for 2-thiolation in <i>M. maripaludis</i>)	Reinforcement of codon–anticodon interaction (<i>E. coli</i> and other mesophiles) Restriction of wobble base pairing (<i>E. coli</i> and other mesophiles) Prevention of frameshift errors (<i>E. coli</i> and other mesophiles) Biosynthesis pathways of xm ⁵ U ₃₄ derivatives are not completely clarified. Although the information on xm ⁵ U ₃₄ derivatives in tRNA from thermophiles is limited, the biosynthesis pathways may be common with those from mesophiles. For the functions and biosynthesis pathways for xm ⁵ U ₃₄ derivatives, see References [121–132,136–139,142]. For the thiolation of xm ⁵ s ² U ₃₄ derivatives, see References [35,133–135]. <i>Aquifex aeolicus</i> exceptionally possesses a DUF752 protein, which is an MT for the xm ⁵ U ₃₄ modifications without an oxidase domain [136]. A mnm ⁵ U nucleoside has been found in modified nucleosides from unfractionated tRNA in several methane archaea [56]. <i>Thermoplasma acidophilum</i> tRNA ^{Leu} possesses ncm ⁵ U ₃₄ [36]. Some thermophiles in Euryarchaea may have a cnm ⁵ U ₃₄ modification in tRNA [137]. The cm ⁵ U ₃₄ formation activity of Elp3 from <i>Methanocaldococcus infernus</i> has been reported [142]. Several related proteins for synthesis of xm ⁵ U ₃₄ derivatives from thermophiles have been used for structural studies [136,138–141].	[34–36,56,121–142]
Cm ₃₄ and cmnm ⁵ Um ₃₄	B	TrmL	Reinforcement of codon–anticodon interaction (<i>E. coli</i>)	[18,143,144]
Gm ₃₄	B	Unknown	Reinforcement of codon–anticodon interaction (<i>G. stearothermophilus</i>)	[19]
Q ₃₄ derivatives	B	Tgt + QueA + QueG	Reinforcement of codon–anticodon interaction (<i>E. coli</i>) Prevention of frame-shift error (<i>E. coli</i>) Biosynthesis pathways and functions of Q derivatives are reviewed in References [152,153]. A crystal structure of QueA from <i>T. maritima</i> has been reported [151].	[20,122,145–153]
Cm ₃₄ and Um ₃₉ (or Cm ₃₉)	A	L7Ae + Nop5 + archaeal fibrillarlin + Box C/D guide RNA (intron)	Reinforcement of codon–anticodon interaction Reinforcement of anticodon-arm In several archaea, an intron in precursor tRNA ^{Trp} functions as a Box C/D guide RNA.	[9,154,155]
Ψ35	A	aPus7 and H/ACA guide RNA system	Reinforcement of codon–anticodon interaction	[46]

Table 1. Cont.

Modified Nucleoside and Position	Distrib.	Modification Enzyme	Predicted Functions and Additional Information	References
m ¹ G ₃₇	B/A	TrmD/Trm5	Prevention of frame-shift error (<i>E. coli</i> and other mesophiles) Recognition by aminoacyl-tRNA synthetase (<i>Saccharomyces cerevisiae</i>)	[36,156–171]
wyosine ₃₇ derivatives	A	Trm5 + Taw1 + Taw2 + Taw3	Reinforcement of codon–anticodon interaction Prevention of the frame-shift error In several archaea, m ¹ G ₃₇ in tRNA ^{Phe} is further modified to wyosine derivatives. For the biogenesis pathway of wyosine derivatives, see References [181–183].	[172–183]
t ⁶ A ₃₇ derivatives	B/A	TsaB, TsaC (TsaC2), TsaD and TsaE/KEOPS complex: Kae1, Bud32, Cgi121 and Pcc1 + Sua5	Reinforcement of codon–anticodon interaction Prevention of frame-shift error Recognition by aminoacyl-tRNA synthetases The biogenesis pathway for t ⁶ A derivatives is reviewed in Reference [191]	[68,184–191]
i ⁶ A ₃₇ derivatives	B	MiaA + MiaB	Prevention of frame-shift error Reinforcement of codon–anticodon interaction Recognition by aminoacyl-tRNA synthetases i ⁶ A derivatives are reviewed in Reference [197]	[10,11,18–20,24,192–197]
m ⁶ A ₃₇	B	YfiC (TrmG?)		[64,198]
Ψ ₃₈ , Ψ ₃₉ and Ψ ₄₀	B/A	TruA/Pus3	Prevention of frame-shift error (<i>E. coli</i>) Reinforcement of anticodon-arm	[10,11,18–20,23,87,199–203]
m ⁷ G ₄₆	B	TrmB	Stabilization of three-dimensional core In the case of <i>T. thermophilus</i> , m ⁷ G ₄₆ modification functions a key factor in a network between modified nucleosides in tRNA and tRNA modification enzymes (see main text) [11]	[10,11,19,67,204–208]
m ⁵ C ₄₈ and m ⁵ C ₄₉	A	archaeal Trm4	Stabilization of three-dimensional core	[9,209,210]
m ⁷ G ₄₉	A	Unknown		[36]
m ⁵ C ₅₁	A	Unknown	Stabilization of T-arm structure	[209]
m ⁵ C ₅₂	A	Unknown	Stabilization of T-arm structure	[209]
Ψ ₅₄ and Ψ ₅₅	A	Pus10	Stabilization of D-arm and T-arm interaction	[211–214]
m ¹ Ψ ₅₄	A	Pus10 + TrmY	Stabilization of D-arm and T-arm interaction	[215–217]

Table 1. Cont.

Modified Nucleoside and Position	Distrib.	Modification Enzyme	Predicted Functions and Additional Information	References
m ⁵ U ₅₄ + m ⁵ s ² U ₅₄	B/A	TrmFO + TtuA + TtuB + TtuC + TtuD + IscS/TrmA + TtuA? + TtuB? + α	Stabilization of D-arm and T-arm interaction (see main text) 2-Thiolation of m ⁵ s ² U ₅₄ in tRNA is reviewed in Reference [239]	[10,11,21–24,67,97,98,134,218–239]
Um ₅₄	A	Unknown	Stabilization of D-arm and T-arm interaction	[44]
Ψ ₅₅	B/A	TruB/Pus10 or archaeal Cbf5 + α	Stabilization of D-arm and T-arm interaction In the case of <i>T. thermophilus</i> , Ψ ₅₅ is required for low-temperature adaptation (see main text) [248].	[10,11,18–20,23,25,36,44,64,67, 211–214,240–248]
Cm ₅₆	A	Trm56	Stabilization of D-arm and T-arm interaction	[9,25,36,44,48,89,249–251]
m ² G ₅₇	A	Unknown		[44,252]
m ¹ I ₅₇	A	archaeal TrmI + unknown deaminase	Stabilization of T-arm structure	[44,253,254]
m ¹ A ₅₇ and m ¹ A ₅₈	A	archaeal TrmI	Stabilization of T-arm structure	[44,255–258]
m ¹ A ₅₈	B	TrmI	Stabilization of T-arm structure	[11,23,67,204,259–264]

This table shows the nucleosides that are modified in tRNA from thermophiles. Most modifications are common to those in tRNA from mesophiles. Several modifications include derivatives and they are summarized as the derivatives (e.g., xm⁵U₃₄ derivatives). In some cases, only modification enzymes from thermophiles have been reported. For example, although Q derivatives have not been confirmed in tRNA from *T. maritima*, the structure of QueA from *T. maritima* has been reported. In these cases, the modifications are listed here. The references for tRNA modifications and tRNA modification enzymes are mainly those for thermophiles. While there are many references for mesophiles, only representative references are cited. Where available, reviews of a modification and related proteins have been cited. Since modified nucleosides in tRNA from thermophilic eukaryotes have not been reported, modified nucleosides in eukaryotic tRNA have not been included here. The following modified nucleosides have been found in unfractionated tRNA from thermophiles. However, their positions and modified tRNA species are unknown: ac⁶A, hn⁶A, ms²hn⁶A, methyl-hn⁶A, m^{2,7}Gm, s²Um, and ac⁴Cm [56,97–100]. Abbreviations are as follows: A, archaea, B, eubacteria, and MT, methyltransferase. The “?” mark indicates the potential function speculated from the structure of the modified nucleosides.

2. Sequencing of tRNA from Thermophiles

The sequence of tRNA provides the most basic information of tRNA. However, as shown in Figure 2, which displays nucleotide sequences of tRNAs from thermophilic eubacteria [10,11,18–24,64,67] and archaea [25,36,44,252], the sequences of only 14 tRNA species have been reported from thermophiles. In addition, in the case of *Aquifex aeolicus* tRNA^{Cys}, the sequence has been only partially determined [24].

In general, sequencing of tRNA from thermophiles is difficult for the following reasons. First, purification of specific tRNA from thermophiles is not easy. Currently, tRNA is purified by the solid DNA probe method [298–300]. In this method, the solid-phase complementary DNA probe is placed in a column and hybridized with the target tRNA and then the target tRNA is eluted from the column. Since the structures of tRNA from thermophiles are very rigid, denaturing the tRNA to allow hybridization is difficult. This problem has been solved by incorporating tetraalkyl-ammonium salt in the hybridization buffer [301]. This salt destabilizes the secondary and tertiary structures of tRNA and promotes formation of the RNA-DNA hetero-duplex. This alteration enabled us to purify *A. aeolicus* tRNA^{Cys} [24], *Thermus thermophilus* tRNA^{Phe} [11], tRNA^{Met}_{f1} [248] and tRNA^{Thr} [263,302], *Thermoplasma acidophilum* initiator tRNA^{Met} [89], elongator tRNA^{Met} [89], and tRNA^{Leu} [36]. Even with the use of tetraalkyl-ammonium salt, however, the solid DNA probe method is not versatile. For example, because the difference between *T. thermophilus* tRNA^{Met}_{f1} and tRNA^{Met}_{f2} is only one G-C base pair in the T-stem (Figure 2H) [21], purification of tRNA^{Met}_{f1} required its separation from tRNA^{Met}_{f2} by BD-cellulose column chromatography before the solid DNA probe method could be applied [248].

Second, since the structure of tRNA from thermophiles is rigid, limited cleavage by formamide [303,304] is difficult. Therefore, it is difficult to apply the classical technique used for RNA sequencing to tRNA from thermophiles. Liquid-chromatography/mass-spectrometry (LC/MS) has been found to be the most reliable method to overcome this problem [305,306]. In general, LC/MS requires prior cleavage of tRNA by RNases. However, because the G-C content in the stem regions of tRNA from thermophiles is very high (Figure 2), RNA fragments with the same sequences are often generated by RNase cleavage. Therefore, use of multiple RNases and/or preparation of gene disruptant strains are required to overcome this problem.

Furthermore, given that it is not possible to distinguish uridine and pseudouridine by MS, cyanoethylation of tRNA is generally required to detect this nucleoside [307]. In the sequencing of *T. acidophilum* tRNA^{Leu} [36], we used a combination of the cyanoethylation and classical formamide method for detection of Ψ₅₄ because the efficiency of cyanoethylation of Ψ₅₄ was low. Thus, specific techniques are required even if an LC/MS system is available.

Third, to determine the modified nucleoside precisely, preparation of a standard compound is often required. For example, it was necessary to prepare the standard ncm⁵U nucleoside from the *Saccharomyces cerevisiae* *trm9* gene disruptant strain [308] to determine the anticodon modification of *T. acidophilum* tRNA^{Leu} [36]. In some cases, synthesis of a standard compound by organic chemistry may be required. Lastly, preparing cultures of thermophiles is not so easy for general biochemical researchers (e.g., under anaerobic conditions at high temperatures).

To overcome these problems, the cooperation of researchers in different fields is required. At present, the solid DNA probe method with tetraalkyl-ammonium coupled with LC/MS is the main method for sequencing tRNA from thermophiles. Therefore, it is anticipated that a large numbers of sequences of tRNA from thermophiles will be reported by using this approach in the future.

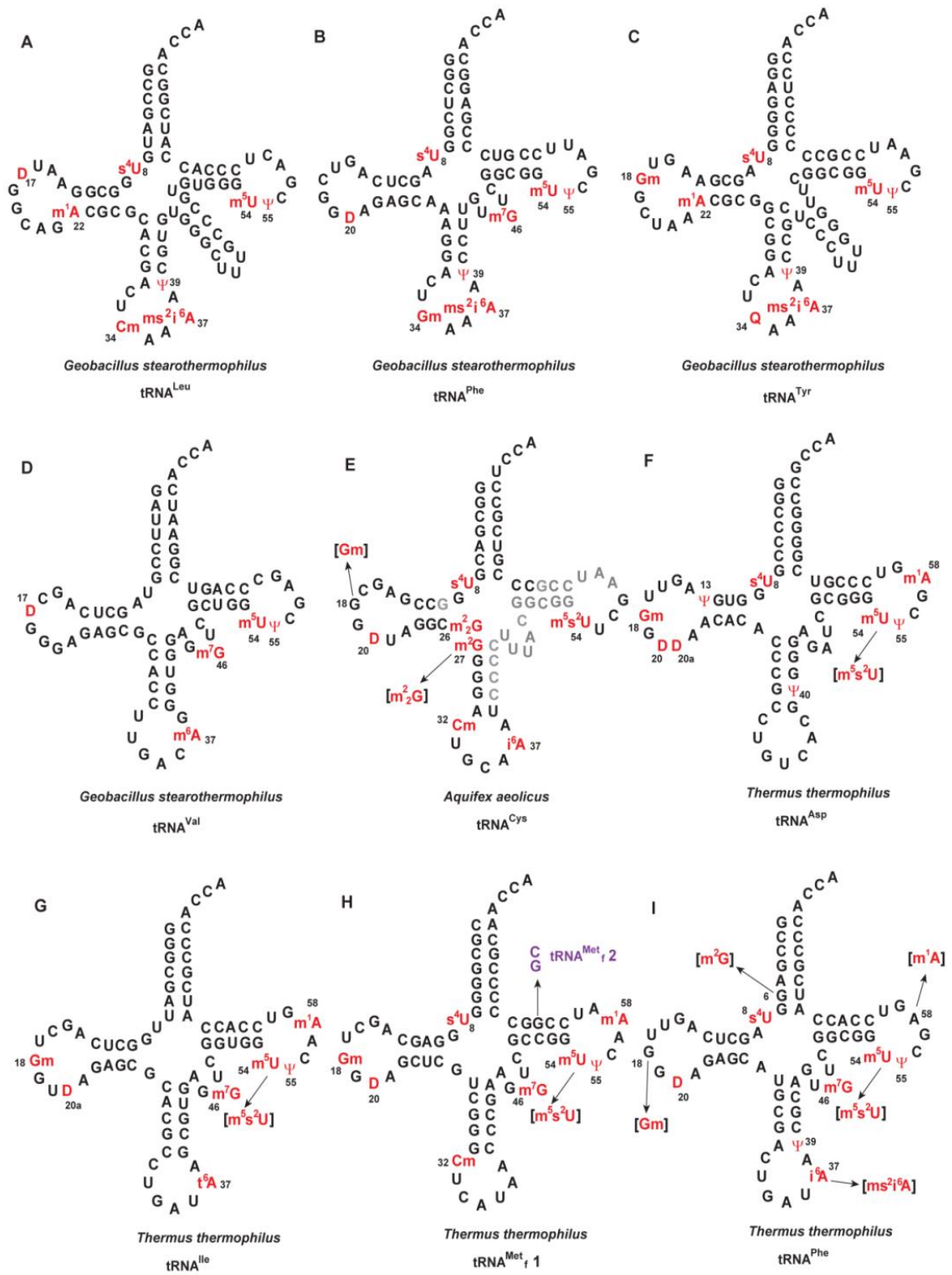


Figure 2. Cont.

example, the degree of 2'-O-methylation in tRNA from *G. stearothermophilus* is increased at high temperatures [309]. Furthermore, several modifications (Gm₁₈, D modifications, and Gm₃₄) in tRNA from *G. stearothermophilus* cannot be explained by the enzymatic activities of the already-known tRNA modification enzymes, which is described in Table 2. Moreover, *T. acidophilum* possesses several distinct tRNA modifications such as G⁺₁₃ and m⁷G₄₉ (Table 2) [36]. Although these differences are present, thermophile-specific modified nucleosides have not been found in tRNA from moderate thermophiles, which suggests that living organisms can survive at 75 °C via the tRNA modifications in mesophiles.

Table 2. Thermophiles: their tRNA modifications and tRNA modification enzymes.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
Moderate Thermophiles			
Eubacteria			
<i>Geobacillus stearothermophilus</i> (<i>Bacillus stearothermophilus</i>) 30–75 °C		Sequences of tRNA ^{Leu} [18], tRNA ^{Phe} [19], tRNA ^{Tyr} [20], and tRNA ^{Val} [64] have been reported (Figure 2). The majority of modifications in tRNA are similar to those in <i>B. subtilis</i> . With increasing culture temperature, the extent of 2'-O-methylation in the tRNA mixture increases [309].	
	Gm ₁₈ (TrmH?)	Although <i>trmH</i> is not encoded in the <i>B. subtilis</i> genome, a <i>trmH</i> -like gene is encoded in the <i>G. stearothermophilus</i> genome. Gm ₁₈ has been found in tRNA ^{Tyr} but not in tRNA ^{Leu} . This modification pattern suggests that the substrate tRNA specificity of <i>G. stearothermophilus</i> TrmH may be different from that of other known TrmH enzymes.	[20]
	D ₁₇ , D ₂₀ and D _{20a} (Dus family protein?)	In <i>G. stearothermophilus</i> tRNA, D ₁₇ , D ₂₀ , and D _{20a} modifications have been reported. In <i>E. coli</i> , three Dus family proteins known as DusA, DusB, and DusC, produce D ₂₀ and D _{20a} , D ₁₇ , and D ₁₆ , respectively [65,66]. In the <i>G. stearothermophilus</i> genome, however, only one gene is annotated as a <i>dus</i> -like gene. Therefore, D modifications in <i>G. stearothermophilus</i> cannot be explained by the tRNA substrate specificity of the known Dus proteins.	[17,19,64]
	m ¹ A ₂₂ (TrmK?)	The m ¹ A ₂₂ modification has been found in tRNA ^{Tyr} and tRNA ^{Ser} from <i>B. subtilis</i> and <i>Mycoplasma capricolum</i> [310,311]. <i>G. stearothermophilus</i> tRNA ^{Leu} and tRNA ^{Tyr} possess m ¹ A ₂₂ [18,20]. The presence of a <i>trmK</i> -like gene in the genome of <i>G. stearothermophilus</i> has been reported [86].	[20,86]
	Gm ₃₄ (unknown MT)	<i>G. stearothermophilus</i> tRNA ^{Phe} possesses Gm ₃₄ (Figure 2B) [19]. In contrast, the nucleoside at position 34 in <i>E. coli</i> tRNA ^{Phe} is unmodified G. Given that <i>E. coli</i> TrmL acts only on tRNA ^{Leu} isoacceptors [143], the 2'-O-methylation of G ₃₄ in tRNA ^{Phe} from <i>G. stearothermophilus</i> is cannot be simply explained by the activity of known TrmL.	[19]
	m ⁶ A ₃₇ (YfiC; TrmG?)		[198]
Archaea			
<i>Methanobacterium thermoaggregans</i> Optimum growth temperature 60 °C		Sequences of tRNA ^{Asn} and tRNA ^{Gly} have been reported [8].	
<i>Methanobacterium thermoautotrophicum</i> 45–75 °C		The modified nucleosides in unfractionated tRNA are essentially common to those in tRNA from mesophilic methane archaea [97].	

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
<i>Methanococcus thermolithotrophicus</i> 17–62 °C		The modified nucleosides in unfractionated tRNA are essentially common to those in tRNA from mesophilic methane archaea [99].	
<i>Thermoplasma acidophilum</i> Optimum growth temperature 55–60 °C		Sequences of tRNA ^{Met} _i [44,252], tRNA ^{Met} _m [25], and tRNA ^{Leu} [36] have been reported. Several recombinant tRNA modification enzymes have been used for biochemical studies.	
	s ⁴ U ₈ and s ⁴ U ₉ (ThiI? + α)	The s ⁴ U ₉ modification has been found in tRNA ^{Leu} [36]. The sulfur donor for s ⁴ U formation is unknown [35].	[36]
	G ⁺ ₁₃ and G ⁺ ₁₅ (ArcTGT + ArcS?)	The G ⁺ ₁₃ modification has been found only in tRNA ^{Leu} from <i>T. acidophilum</i> [36]. ArcTGT from <i>T. acidophilum</i> acts on both G13 and G15 in tRNA ^{Leu} [47].	[36,47]
	m ² ₂ G ₂₆ (Trm1)		[89]
	ncm ⁵ U ₃₄ (Elp3?)		[36]
	m ¹ G ₃₇ (Trm5)		[89]
	m ⁷ G ₄₉ (unknown MT)		[36]
	Cm ₅₆ (Trm56)	The presence of unusual <i>trm56</i> -like gene in the <i>T. acidophilum</i> genome has been reported in a bioinformatics study [250]. The Trm56 enzymatic activity has been confirmed via the recombinant protein [89]. <i>T. acidophilum</i> Trm56 exceptionally possesses a long C-terminal region in the SPOUT tRNA MT [312].	[89,250,312]
Extreme-thermophiles and Hyper-thermophiles			
Eubacteria			
<i>Aquifex aeolicus</i> Optimum growth temperature 85–94 °C		The partial sequence of tRNA ^{Cys} has been reported [24] (Figure 2E). Several tRNA MT activities have been detected in the <i>A. aeolicus</i> cell extract using an <i>E. coli</i> tRNA mixture [24]. The tRNA modification enzymes listed below were characterized via recombinant proteins.	
	Gm ₁₈ (TrmH)		[74,77]
	D ₂₀ (Dus)	D ₂₀ exists in tRNA ^{Cys} . However, the nucleoside at position 20a is unmodified U [24]. Therefore, <i>A. aeolicus</i> Dus may act only on U ₂₀ .	[24,82]
	m ² G ₂₆ , m ² ₂ G ₂₆ , m ² G ₂₇ and m ² ₂ G ₂₇ (Trm1)	<i>Aquifex aeolicus</i> exceptionally possesses Trm1 in eubacteria [24].	[24,95]
	I ₃₄ (TadA)		[104,105]

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
	mnm ⁵ U ₃₄ (MnmC2)	MnmC catalyzes the final methylation step of mnm ⁵ U synthesis. <i>Aquifex aeolicus</i> MnmC2 comprises only an MT domain.	[136]
	(MnmD; previously called GidA)		[140,141]
	k ² C ₃₄ (TilS)		[111–113]
	m ¹ G ₃₇ (TrmD)	The dimer structure of <i>A. aeolicus</i> TrmD is stabilized by inter-subunit disulfide bonds [165].	[160,162,165]
	m ⁷ G ₄₆ (TrmB)	TrmB proteins from thermophiles (<i>A. aeolicus</i> and <i>T. thermophilus</i>) possess a long C-terminal region.	[206–208]
	m ⁵ U ₅₄ and m ⁵ s ² U ₅₄ (TrmFO)	The presence of <i>trmFO</i> gene in <i>A. aeolicus</i> genome was initially described in Reference [221].	[24,221]
	m ¹ A ₅₈ (TrmI)		[257,262]
<i>Thermotoga maritima</i> 80–90 °C		Sequences of tRNA from <i>T. maritima</i> have not been reported. Recombinant proteins have been used for biochemical and structural studies.	
	hn ⁶ A (?)	hn ⁶ A was first identified in modified nucleosides from unfractionated tRNA from <i>T. maritima</i> [313]. Because hn ⁶ A was subsequently found in modified nucleosides from psychrophilic archaea [56], it is not a thermophile-specific modification. <i>Thermotoga maritima</i> and <i>Thermodesulfobacterium commune</i> exceptionally possess hn ⁶ A in eubacteria. The modification position in tRNA, modified tRNA species, and biosynthesis pathway of hn ⁶ A are unknown.	[56,313]
	s ⁴ U ₈ (ThiI + IscS)		[31,32]
	oQ ₃₄ (QueA)		[151]
	mnm ⁵ U ₃₄ (TrmE)		[138,139]
	t ⁶ A ₃₇ (TsaB, TsaC/TsaC2, TsaD and TsaE)		[190]
	ms ² i ⁶ A ₃₇ (MiaB)		[194–196]
	m ¹ G ₃₇ (TrmD)		[171]
	m ⁵ U ₅₄ and m ⁵ s ² U ₅₄ (TrmFO and TtuA)	The m ⁵ s ² U nucleoside has been found in unfractionated tRNA from <i>T. maritima</i> [97].	[97,134,221,222]
	Ψ ₅₅ (TruD)		[244–247]

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
	m ¹ A ₅₈ (TrmI)		[257]
<i>Thermodesulfobacterium commune</i> Optimum growth temperature 70 °C	hn ⁶ A and ms ² hn ⁶ A (?)	hn ⁶ A and ms ² hn ⁶ A have been found in modified nucleosides from unfractionated tRNA from <i>T. commune</i> . The ms ² hn ⁶ A modification may be derived from hn ⁶ A. So far, <i>T. commune</i> is the only eubacterium that possesses ms ² hn ⁶ A in tRNA. The modification position in tRNA, modified tRNA species, and biogenesis pathway of hn ⁶ A and ms ² hn ⁶ A are unknown.	[313]
<i>Thermus flavus</i> Optimum growth temperature 70 °C		Partial purification of tRNA m ¹ A ₅₈ MT has been reported: the activity of tRNA m ⁷ G ₄₆ MT has also been described [204].	
<i>Thermus thermophilus</i> 50–83 °C		Sequences of tRNA ^{Met} f1 [21], tRNA ^{Met} f2 [21], tRNA ^{Ile} 1 [67], tRNA ^{Asp} [23], and tRNA ^{Phe} [10,11] have been reported (Figure 2). Partial sequences of tRNA ^{Ser} _{GGA} [259], tRNA ^{Pro} _{GGA} [314], and tRNA ^{Pro} _{GGA} [314] have been determined. The modification extent of Gm ₁₈ , m ⁵ s ² U ₅₄ and m ¹ A ₅₈ changes with the culture temperature. At high temperatures (>75 °C), m ⁷ G ₄₆ [11], m ⁵ s ² U ₅₄ [230], and m ¹ A ₅₈ [260] modifications are essential for survival. At low temperatures (<55 °C), Ψ ₅₅ is essential for survival [248] and m ⁵ U ₅₄ supports this effect [225] (see the main text). Recombinant proteins have been used for biochemical and structural studies.	
	m ² G ₆ (TrmN)		[10,11,13–15]
	Gm ₁₈ (TrmH)		[10,11,21,23,30,69–73,75,76,78–81]
	D ₂₀ and D _{20a} (DusA)		[10,11,23,67,83–85]
	Cm ₃₄ and cmnm ⁵ Um ₃₄ (TrmL)		[144]
	Ψ ₃₉ and Ψ ₄₀ (TruA)		[10,11,18,23,202]
	m ⁷ G ₄₆ (TrmB)		[10,11,21,23]
	m ⁵ U ₅₄ and m ⁵ s ² U ₅₄ (TrmFO + TtuA + TtuB + TtuC + TtuD + IscS)		[10,11,17,21,23,67,218–236,239]
	Ψ ₅₅ (TruB)		[10,11,21,23,67,248]
	m ¹ A ₅₈ (TrmI)		[11,30,257,259–261,263,264]

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
Archaea			
<i>Aeropyrum pernix</i> 80–100 °C	Ψ ₁₃ and Ψ ₁₅ (archaeal Pus7 and H/ACA guide RNA system)	A guide RNA for Ψ formation has been predicted based on genome sequencing.	[46]
<i>Archaeoglobus fulgidus</i> 60–95 °C		Modified nucleosides in unfractionated tRNA from <i>A. fulgidus</i> have been reported [97].	
	agm ² C ₃₄ (TiaS)		[116,118,119,315]
<i>Methanocaldococcus igneus</i> (<i>Methanococcus igneus</i> ; <i>Methanotorris igneus</i>) 45–91 °C		Modified nucleosides in unfractionated tRNA from <i>M. igneus</i> have been reported [56,99].	
<i>Methanocaldococcus infernus</i> 55–92 °C	cm ⁵ U ₃₄ (Elp3)		[142]
<i>Methanocaldococcus jannashii</i> (<i>Methanococcus jannashii</i>) 48–94 °C		Although sequences of tRNA are unknown, the recombinant proteins listed below have been used for biochemical and structural studies.	
	m ² G ₆ (Trm14)		[12]
	G ⁺ ₁₅ (ArcTGT + ArcS)		[51,59]
	Cm ₃₄ and Um ₃₉ (L7Ae, Nop5, aFib, Box C/D guide RNA system)		[316]
	m ¹ G ₃₇ (Trm5)		[159,161,163,164,166–170]
	imG ₂₃₇ (Trm5b + Taw1)		[173,179]
	yW-86 ₃₇ (Taw2)		[174]
	m ⁵ C ₄₈ and m ⁵ C ₄₉ (archaeal Trm4)		[210]
	Ψ ₅₄ and Ψ ₅₅ (Pus10)		[211–214]
	m ¹ Ψ ₅₄ (Pus10 + TrmY)		[215–217]
	Ψ ₅₅ (archaeal Cbf5)		[240]
<i>Methanopyrus kandleri</i> 84–110 °C (Strain 116: up to 122 °C)		Many unique modified nucleosides have been found in unfractionated tRNA [100]. tRNAs likely contain many 2'-O-methylated nucleosides derived from the C/D box guide RNA system [17].	

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
	ac ⁶ A (?)	The ac ⁶ A nucleoside has been purified from the modified nucleosides in unfractionated tRNA and its structure determined. The modification site, modified tRNA species, and biosynthesis pathway are unknown.	[100]
	U ₈ (CDAT8)		[16]
<i>Methanothermus fervidus</i> 80–97 °C		Only tRNA genes were reported in an early study [317].	
<i>Nanoarchaeum equitans</i> 70–98 °C		A unique tRNA processing system has been found [318,319]. The processing of small RNAs in <i>N. equitans</i> is reviewed in Reference [320].	
	m ¹ G ₃₇ and imG ₂₃₇ (Trm5a)		[176]
	m ⁵ U ₅₄ (TrmA-like protein)		[237]
<i>Pyrobaculum aerophilum</i> Optimum growth temperature 100 °C	Cm ₅₆ (L7Ae, Nop5, aFib, Box C/D guide RNA system)	Cm ₅₆ in tRNA is generally produced by Trm56. However, this modification in <i>P. aerophilum</i> is synthesized by the C/D box guide RNA system.	[249]
<i>Pyrobaculum caldifontis</i> 90–95 °C	G ⁺ ₁₅ (ArcTGT + QueF-like protein)	Eubacterial QueF catalyzes the conversion from preQ ₀ to preQ ₁ . In <i>P. caldifontis</i> , however, QueF-like protein catalyzes the conversion from preQ ₀ at position 15 in tRNA to G ⁺ ₁₅ .	[60,62,63]
<i>Pyrobaculum islandicum</i> Optimum growth temperature 100 °C		Modified nucleosides in unfractionated tRNA from <i>P. islandicum</i> have been reported [97].	
<i>Pyrococcus abyssi</i> Optimum growth temperature 96 °C		No tRNA sequence has been determined. However, the tRNA modification enzymes listed below have been characterized.	
	m ² G ₁₀ and m ² ₂ G ₁₀ (archaeal Trm11, Trm-G10 enzyme, Trm-m22G10 enzyme)		[40,41]
	Ψ ₁₃ and Ψ ₃₅ (archaeal Pus7 and H/ACA guide RNA system)		[46]
	Cm ₃₄ and Um ₃₉ (L7Ae, Nop5, aFib, and C/D box guide RNA system)	Cm ₃₄ and Um ₃₉ in tRNA ^{Trp} are formed by the C/D box guide RNA system in which the intron functions as a guide RNA.	[154,155]
	m ¹ G ₃₇ (Trm5b)		[180]
	m ¹ G ₃₇ and imG ₂₃₇ (Trm5a)		[176,177,179]

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
	imG-14 ₃₇ (Taw1)		[173,175]
	t ⁶ A ₃₇ (Kae1)		[185]
	(KEOPS complex)		[184]
	(Sua5 + KEOPS complex)		[187,189]
	m ⁵ C ₄₈ and m ⁵ C ₄₉ (archaeal Trm4 + archaese)		[209]
	m ⁵ U ₅₄ (TrmA-like protein, PAB0719)		[237,238]
	Ψ ₅₅ (Cbf5 + Nop10)		[241]
	Cm ₅₆ (Trm56)		[249]
	m ¹ A ₅₇ and m ¹ A ₅₈ (archaeal TrmI)		[255–258]
<i>Pyrococcus furiosus</i> Optimum growth temperature 100 °C		Modified nucleosides in unfractionated tRNA from <i>P. furiosus</i> have been reported [98]. Activity of several tRNA modification enzymes has been detected in the cell extract of <i>P. furiosus</i> [9].	
	m ² G ₆ (Trm14)		[13,15]
	m ² G ₁₀ and m ² ₂ G ₁₀ (archaeal Trm11, Trm-G10 enzyme, Trm-m22G10 enzyme)		[42]
	G ⁺ ₁₅ (ArcTGT)		[57]
	m ² G ₂₆ and m ² ₂ G ₂₆ (Trm1)		[91,92]
	t ⁶ A ₃₇ (KEOPS complex)		[188]
	Ψ ₅₄ and Ψ ₅₅ (Pus10)		[212,214]
	Ψ ₅₅ (Cbf5 + Nop10 + Gar1)		[242]
<i>Pyrococcus horikoshii</i> 80–102 °C		The crystal structure of Nop5 in the C/D box guide RNA system from <i>P. horikoshii</i> has been solved [321].	
	G ⁺ ₁₅ (ArcTGT)		[50,52–55,89]
	m ² G ₂₆ and m ² ₂ G ₂₆ (Trm1)		[89,93]

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
	yW-86 ₃₇ (Taw2)		[174]
	m ⁵ s ² U ₅₄ (TtuA)		[233]
	Cm ₅₆ (Trm56)		[251]
<i>Pyrodictium occultum</i> Optimum growth temperature 105 °C		Modified nucleosides in unfractionated tRNA have been analyzed and many 2'-O-methylated nucleosides found [97,98]. mimG was originally found among the modified nucleosides in tRNAs from <i>P. occultum</i> , <i>Sulfolobus solfaraticus</i> , and <i>Thermoproteus neutrophilus</i> [322]. Although the melting temperature of <i>P. occultum</i> tRNA ^{Met} i transcript is only 80 °C and that of native tRNA ^{Met} i is more than 100 °C (see main text) [323].	
<i>Pyrolobus fumarii</i> This archaeon can survive at 113 °C.		Modified nucleosides in unfractionated tRNA have been analyzed [324].	
<i>Stetteria hydrogenophila</i> Optimum growth temperature 95 °C		Modified nucleosides in unfractionated tRNA have been analyzed and methyl-hn ⁶ A, ms ² hn ⁶ A, and m ² . ⁷ Gm identified [56].	
<i>Sulfolobus acidocaldarius</i> Optimum growth temperature 75–80 °C		Sequence of tRNA ^{Met} i has been reported [44]. The m ¹ I ₅₇ modification was originally found in tRNAs from <i>S. acidocaldarius</i> and <i>Haloferax volcanii</i> [253]. G ⁺ was first isolated from the nucleosides in <i>S. acidocaldarius</i> tRNAs and its structure determined [49]. The structures of wyosine derivatives (imG-14 and imG2) have been determined by using the nucleosides from <i>S. acidocaldarius</i> tRNAs [325].	
	m ¹ A ₉ (archaeal Trm10)		[37,38]
	Ψ ₁₃ and Ψ ₃₅ (archaeal Pus7 and H/ACA guide RNA system)		[46]
	Cm ₃₂ (archaeal TrmJ)		[96]
<i>Sulfolobus solfaraticus</i> 55–90 °C		mimG was originally found among the modified nucleosides in tRNAs from <i>P. occultum</i> , <i>S. solfaraticus</i> , and <i>Thermoproteus neutrophilus</i> [322]. The structure of box C/D RNP from <i>S. solfaraticus</i> has been reported [326].	
	agm ² C (TiaS)	The identification of agm ² C ₃₄ in <i>Haloarcula marismortui</i> tRNA ^{Ile} 2 and the presence of agm ² C in <i>S. solfaraticus</i> tRNA have been reported.	[117]
	Ψ ₁₃ and Ψ ₃₅ (archaeal Pus7 and H/ACA guide RNA system)	Generally, Ψ ₃₅ in tRNA ^{Tyr} is synthesized by archaeal Pus7. However, Pus7 from <i>S. solfaraticus</i> possesses weak Ψ ₁₃ formation activity but not Ψ ₃₅ formation activity. In <i>S. solfaraticus</i> and <i>A. pernix</i> , a guide RNA for Ψ ₃₅ formation exists.	[46]

Table 2. Cont.

Species	Predicted Enzyme	Distinct tRNA Modifications and General Information	References
	imG ₂₃₇ (Trm5a; SSO2439 protein)	Trm5a (SSO2439 protein) does not possess m ¹ G ₃₇ formation activity and is used only for imG ₂ formation.	[178]
	mimG ₃₇ (Taw3)		[180]
<i>Sulfolobus tokodaii</i> This archaeon can survive at 87 °C.	Ψ ₁₃ and Ψ ₃₅ (archaeal Pus7 and H/ACA guide RNA system)		[46]
	t ⁶ A ₃₇ (Sua5)		[327–329]
<i>Thermococcus celer</i> This archaeon can survive at 85 °C.		Although tRNA genes were analyzed in an early study [330], there is no information on tRNA modifications.	
<i>Thermococcus kodakarensis</i> (<i>Thermococcus kodakaraensis</i> ; <i>Pyrococcus kodakarensis</i>) 65–100 °C	m ¹ A ₉ and m ¹ G ₉ (archaeal Trm10)		[37,39]
	m ² G ₁₀ and m ² ₂ G ₁₀ (archaeal Trm11, Trm-G10 enzyme, Trm-m22G10 enzyme)		[43]
	G ⁺ ₁₅ (ArcTGT)		[47]
	m ⁵ U ₅₄ (TrmA-like protein)		[237]
<i>Thermoproteus neutrophilus</i> Optimum growth temperature 85 °C		Modified nucleosides in unfractionated tRNA have been analyzed [97]. mimG was originally found among the modified nucleosides in tRNAs from <i>P. occultum</i> , <i>S. solfaraticus</i> , and <i>T. neutrophilus</i> [322].	

Only distinct modifications that have been investigated are listed by thermophile species. In many cases, only tRNA modification enzymes (rather than modifications) have been studied by using recombinant proteins. For example, the presence of the m⁷G₄₆ modification has not been confirmed in tRNA from *A. aeolicus*, but TrmB (tRNA m⁷G₄₆ MT) has been characterized through the recombinant protein. In this case, m⁷G₄₆ (TrmB) is listed in the section “*Aquifex aeolicus*”. The moderate thermophiles and extreme-thermophiles along with hyper-thermophiles are separated. Transfer RNA modifications in thermophilic eukaryotes are unknown. Abbreviation: MT, methyltransferase.

4. Strategies of tRNA Stabilization by Modified Nucleosides in Extreme-Thermophiles and Hyper-Thermophiles

In general, the G-C content in the stem regions of tRNA from thermophiles is very high (Figure 2). However, the stability of tRNA from thermophiles cannot be explained only by the increase in G-C content in the stem region. For example, although the melting temperature of *T. thermophilus* tRNA^{Phe} transcript is 76 °C, that of the native tRNA^{Phe} is 84.5 °C [11]. Thus, modified nucleosides are essentially required for stabilization of tRNA at high temperatures. Modified nucleosides in tRNA from thermophiles have been studied mainly from the view point of tRNA stabilization. So far, only a few modified nucleosides specific to thermophiles have been found (Figure 3). These thermophile-specific modified nucleosides seem to stabilize the tRNA structures at high temperatures. As described below, extreme-thermophiles and hyper-thermophiles possess two strategies of tRNA stabilization by modified nucleosides. One is based on thermophile-specific modification such as m⁵s²U₅₄ (Figure 3A) and the other is based on 2'-O-methylations at multiple positions in tRNA (Figure 3B–E). Recently, the unknown modified nucleoside at position 26 in *Sulfolobus acidocaldarius* tRNA^{Met} (Figure 2M) was described as m²₂Gm [96]. On the whole, however, the modification site(s), modified tRNA species, and biosynthesis pathways of most thermophile-specific modified nucleosides are unknown. Moreover, these nucleosides may have additional functions at high temperatures beyond their structural effect.

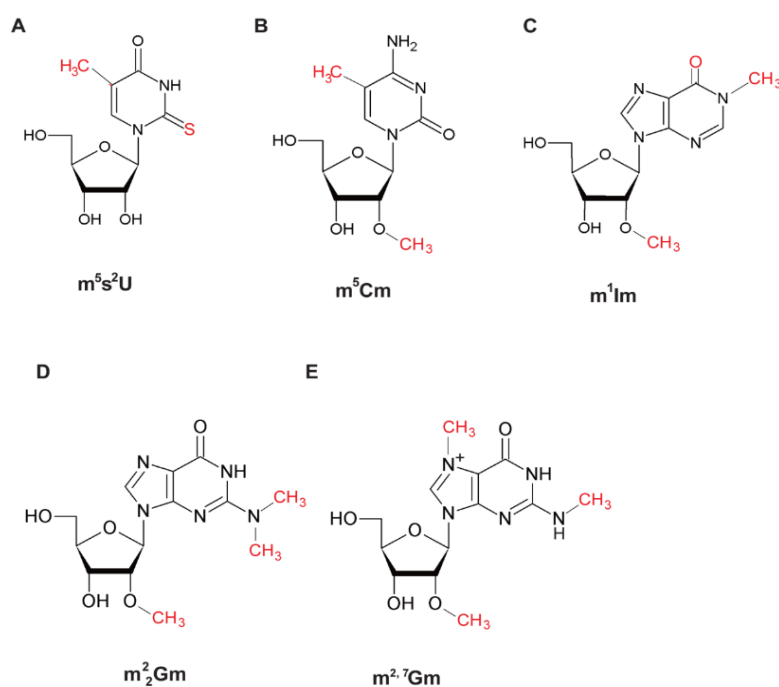


Figure 3. Thermophile-specific modified nucleosides in tRNA. Abbreviations of modified nucleosides are given in Supplementary Table S1. (A) m⁵s²U. (B) m⁵Cm. (C) m¹Im. (D) m²₂Gm. (E) m^{2.7}Gm. The modifications are indicated in red.

4.1. m⁵s²U₅₄ Is a Typical Thermophile-Specific Modified Nucleoside in tRNA

The m⁵s²U₅₄ modification was originally found in tRNA from *T. thermophilus* [331]. Subsequently, this modified nucleoside was found in tRNA from *A. aeolicus*, *T. maritima*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, and *T. kodakarensis* (Table 2) but not from mesophiles. The m⁵s²U₅₄ modification forms a reverse Hoogsteen base-pair with A₅₈ (or m¹A₅₈) in tRNA and stabilizes the tRNA structure by stacking with the G₅₁–C₆₁ base-pair [220]. Because the 2-thio-modification at position 54 increases the melting temperature of tRNA by more than 3 °C [22,218,220], the m⁵s²U₅₄ modification contributes to stabilization of the tRNA structure. The degree of m⁵s²U₅₄ modification increases with an increasing temperature [22,67,220,229]. At 80 °C, the extent of m⁵s²U₅₄ modification in tRNA is

almost 100% [22,67,220,229]. The melting temperature of tRNA mixture is maintained above 85 °C due to the presence of m⁵s²U₅₄ modification [229] and *T. thermophilus* can grow at 50 to 83 °C. Thus, living organisms can survive at 80 °C due to the presence of m⁵s²U₅₄ modification in tRNA.

4.2. The Network Between Modified Nucleosides in tRNA and tRNA Modification Enzymes in *T. thermophilus* Adapts Protein Synthesis at Low and High Temperatures

Under natural conditions, the temperature of hot spring water fluctuates for several reasons including an influx of river water, snowfall, and an eruption of hot water. In accordance with these temperature changes, *T. thermophilus* can synthesize proteins efficiently at a wide range of temperatures (50 to 83 °C) by regulating the flexibility (rigidity) of its tRNA [220]. At high temperatures (above 75 °C), three modified nucleosides in tRNA, m⁵s²U₅₄ [230], m¹A₅₈ [260], and m⁷G₄₆ [11] are essential for survival of *T. thermophilus*. The m¹A₅₈ modification is one of the positive determinants for the two-thiolation system of m⁵s²U₅₄. Thus, a *T. thermophilus* disruptant strain of the *trmI* gene encoding the tRNA m¹A₅₈ methyltransferase cannot grow at 80 °C [229,260]. The presence of m⁷G₄₆ modification in tRNA increases the speed of tRNA modification enzymes such as TrmH for Gm₁₈, TrmD for m¹G₃₇, and TrmI for m¹A₅₈ [11]. The m¹A₅₈ modification further increases the rate of sulfur-transfer to m⁵U₅₄ by the 2-thiolation system and the introduced modified nucleosides coordinately stabilize the tRNA structure. Thus, the m⁷G₄₆ modification produced by TrmB is a key factor in the network between modified nucleosides in tRNA and tRNA modification enzymes of *T. thermophilus* at high temperatures. In the *trmB*-gene disruptant strain, tRNA^{Phe} and tRNA^{Ile} were found to be degraded by a temperature shift from 70 °C to 80 °C and heat-shock proteins were not synthesized efficiently [11].

At low temperatures (below 55 °C), in contrast, the Ψ₅₅ modification produced by TruB is essential for the survival of *T. thermophilus* [248]. The presence of Ψ₅₅ stabilizes both the local structure of the T-arm and the interaction of the T-arm with the D-arm in tRNA. The local rigidity in tRNA caused by Ψ₅₅ slows down the speeds of introducing modified nucleosides around Ψ₅₅ (Gm₁₈, m⁵s²U₅₄ and m¹A₅₈), which maintains the flexibility of tRNA at low temperatures. The presence of m⁵U₅₄ modification by TrmFO supports this effect of Ψ₅₅ [225].

It should be mentioned that D modifications are thought to bring flexibility to tRNA because D does not stack with other bases and brings about the C2'-endo form of ribose [332]. However, a *T. thermophilus* disruptant strain of the *dusA* gene encoding tRNA D₂₀/D_{20a} synthase did not show growth retardation at 50, 60, 70, or 80 °C, and abnormal modifications were not observed in tRNA from this strain [85]. Therefore, the function of D₂₀ and D_{20a} modifications is unknown. Since DusA recognizes the interaction of T-arms and D-arms in tRNA [84], the stabilization of the L-shaped tRNA structure by other modified nucleosides is required for the efficient introduction of D₂₀ and D_{20a} at high temperatures [85]. Thus, D₂₀ and D_{20a} are relatively late modifications in *T. thermophilus* tRNA.

Although the above network is a temperature adaptation system of *T. thermophilus*, it regulates the order in which modified nucleosides are introduced into tRNA. Similar networks have been found in mesophiles [333]. In *Escherichia coli*, for example, the 2'-O-methylation at position 34 by TrmL requires an i⁶A₃₇ modification [334]. However, the network in *T. thermophilus* is distinct because it regulates the structure of a three-dimensional core and many modifications in tRNA are related. One of the advantages of this system is that protein synthesis is not required. The response of the system is very rapid. It is possible that thermophilic archaea possess a similar network between modified nucleosides in tRNA and tRNA modification enzymes because some of them can also grow at a wide range of temperatures.

4.3. Stabilization of tRNA Structure by 2'-O-Methylation

Because 2'-O-methylation shifts the equilibrium of ribose puckering to the C3'-endo form and enhances the hydrophobic interaction, this modification, when carried out at multiple positions, brings rigidity of tRNA. Furthermore, 2'-O-methylations prevents hydrolysis of phosphodiester-bonds in tRNA at high temperatures. Therefore, 2'-O-methylations may prolong the half-lives of tRNA. Notably, there

is a living organism in which tRNA is stabilized without $m^5s^2U_{54}$ modification. A hyper-thermophilic archaeon, *Pyrodicticum occultum* can grow at 105 °C, and various 2'-O-methylated nucleosides such as Ψm , m^1Im , and m^2_2Gm are present in its tRNA, but s^2U and m^5s^2U are not observed [97,98]. Notably, although the melting temperature of the *P. occultum* tRNA^{Met} transcript is 80 °C, that of the native tRNA^{Met} is more than 100 °C [323]. Thus, the melting temperature of *P. occultum* tRNA is increased by more than 20 °C through a combination of numerous 2'-O-methylated nucleosides.

Methanopyrus kandleri can grow at more than 110 °C and tRNAs from this archaeon contain many unique modifications such as U_8 (the product of C_8 to U_8 editing) [16], ac^6A , $m^{2,7}Gm$, and methyl-hn⁶A [100]. Furthermore, *M. kandleri* possesses 132 species of C/D-box guide RNAs [17], which suggests that RNAs are highly methylated by the L7Ae, Nop5, aFib, and C/D-box guide RNA system. In the case of *M. kandleri*, therefore, tRNA seems to be stabilized by unique modifications and 2'-O-methylations.

These observations suggest that living organisms can survive at more than 100 °C by a combination of 2'-O-methylations and other thermophile-specific tRNA modifications.

4.4. Other tRNA Stabilization Factors

RNA binding proteins, polyamines, magnesium ions, and potassium ions are all able to stabilize tRNA in thermophiles. For example, transfer RNA-binding protein 111 (Trbp111) is an RNA-binding protein that is observed only in *A. aeolicus* [335–337]. *A. aeolicus* can grow at 94 °C and modified nucleosides in tRNA of this hyperthermophilic eubacterium are not so different from those in tRNA from *T. thermophilus*, which grows at temperatures below 83 °C. Therefore, Trbp111 may provide more than 10 °C of tRNA stabilization in *A. aeolicus*. The docking model of Trbp111 and tRNA suggests that Trbp111 stabilizes the three-dimensional core of tRNA [336]. Archease is another tRNA-binding protein that can change the methylation site of *P. abyssi* Trm4 [209]. Furthermore, archease promotes the ligation of tRNA exons during tRNA splicing [338,339]. Therefore, it has the potential to stabilize the tRNA structure at high temperatures.

Many tRNA-binding proteins and RNA chaperone proteins have been identified in eukaryotic cells [340,341]. Although these types of protein are unknown in thermophilic eukaryotes, some of them may stabilize the tRNA structure (or help correct folding of tRNA) at high temperatures. Recently, it was revealed that *E. coli* TruB (tRNA Ψ_{55} synthase [243]) possesses an RNA chaperone activity [342,343]. In the case of *T. thermophilus*, although the Ψ_{55} modification is required for survival at low temperatures (below 55 °C), the *truB* gene disruptant strain shows abnormal growth at 80 °C [248]. Therefore, the RNA chaperone effect of TruB may also be expressed at high temperatures in *T. thermophilus*. Furthermore, these observations suggest that other tRNA modification enzymes have the potential to work as RNA chaperones.

In general, polyamines have the potential to interact with nucleic acids and phospholipids because they possess multiple positive charges and hydrophobic areas. There are several studies on the interaction between tRNA and polyamines [344–347]. Thermophiles produce unique polyamines including long and branched polyamines [348–351]. Therefore, polyamines probably contribute to stabilize the tRNA structure at high temperatures. Furthermore, in vitro studies have shown that thermophile-specific long and branched polyamines affect the activities of several tRNA modification enzymes [81,352]. For example, TrmH from *T. thermophilus* methylates tRNA transcript at 80 °C only in the presence of long or branched polyamines [81]. Moreover, the long and branched polyamines are required for the maintenance of several tRNAs and the 70S ribosome and are essential for the survival of *T. thermophilus* at high temperatures [353].

Lastly, magnesium ions have been shown to be a tRNA stabilization factor [6,88,354] and are very important when considering the structural effects of several modified nucleosides in tRNA [58,88,354–356]. However, the precise concentration of magnesium ions in thermophile cells is unknown. It may differ depending on the growth environments. Potassium ions also function as RNA stabilization factor [88]. Notably, the intercellular concentration of some hyperthermophilic archaea

(*M. ferrovidus* and *P. furiosus*) is much higher (700–900 mM) than that of mesophilic archaea [357]. In the case of *Methanothermobacter sociabilis*, the intercellular potassium concentration reaches 1060 mM [357]. These high concentrations of potassium ions may have effects on the stability of tRNA and the activities of tRNA modification enzymes.

5. tRNA Modifications and Environmental Stresses at High Temperatures

Recent studies have revealed that the modifications in tRNA are stress-resistance and/or stress-response factors [102,358–361]. Furthermore, a high temperature itself can be a stress factor for living organisms because some modified nucleosides (D and m⁷G) are liable at high temperatures [297].

5.1. Oxidative Stress

Many thermophiles can grow under aerobic conditions. For example, *Aeropyrum pernix* can grow at 100 °C under aerobic conditions. Under such conditions at high temperatures, living organisms seem to be exposed to heavy oxidative stress, which is a typical environmental stress. The amount of antioxidant enzymes such as superoxide-dismutase, catalase, and peroxidase in *Thermus filiformis*, which is an extreme-thermophilic eubacterium, increases at high temperatures [362].

Among tRNA modification enzymes, both Fe-S cluster proteins [34,130,134,142,150,173,196,236,363] for sulfur-transfer, reduction of base and/or radical S-adenosyl-l-methionine (SAM) reaction, and enzymes with catalytic cysteine residues [141,210,364–366], seem to be easily changed under oxidative stress. In some cases, the substrate (e.g., electron donors and folate derivatives [126,221,227,367]) may be unstable under aerobic conditions at high temperatures. Similarly, several modified nucleosides such as D and s⁴U may be labile under oxidative stress at high temperatures. Therefore, aerobic thermophiles need to protect their cellular components from oxidative stress and their tRNA modifications may respond to such stress as in mesophiles. Overall, however, the relationship between oxidative stress and tRNA modifications in thermophiles is unclear. In addition, tRNA modification systems in some thermophiles may utilize aerobic conditions at high temperatures. For example, *A. aeolicus* grows under microaerophilic conditions at high temperatures (80–94 °C) and the dimer structure of *A. aeolicus* TrmD is stabilized by inter-subunit disulfide bonds [165].

5.2. Other Environmental Stresses

Thermophiles often live in severe environments such as extreme pH and high pressure in addition to high temperatures. These environmental stresses may give rise to the diversity of tRNA modifications. At present, however, there are no data to support this viewpoint.

UV-stress is one such environmental stress and the s⁴U modification in tRNA is a known UV-stress-resistance factor for *E. coli* [368] and *Salmonella typhimurium* [27]. Thus, the s⁴U modification in tRNA is likely to work similarly to a UV-resistant factor in thermophiles. Interestingly, the genomes of *Archaeoglobus fulgidus* and *Methanocaldococcus janaschii*, which were isolated from the oil mines under the sea and deep sea, respectively, contain a *thiI* genes [369] (AF_RS04455 and MJ_RS04985, respectively) encoding tRNA s⁴U₈ synthetase. Since sunlight does not reach the environments in which these thermophilic archaea live, the s⁴U modification and/or ThiI may have an additional function (e.g., sulfur-metabolism) in these archaea. Furthermore, it was recently reported that the melting temperature of tRNA from an *E. coli thiI*-gene disruptant strain was decreased relative to the wild-type strain [33]. Therefore, the s⁴U₈ modification may contribute to stabilize tRNA structure. Furthermore, UV-stress may have an effect on other tRNA modifications via the cross-linking of s⁴U in tRNA. For example, the methylation speed of *T. thermophilus* TrmH is decreased when the substrate tRNA is cross-linked [30].

Lastly, the availability of nutrient-factors may have an effect on tRNA modifications in thermophiles. To test this idea, the extent of modifications in tRNA from *T. thermophilus* cells cultured in a nutrient-poor condition was investigated [227]. Contrary to expectation, the extent of the modification of all methylated nucleosides analyzed was normal, which demonstrates that the limited nutrients

were preferentially consumed in the tRNA modification systems [227]. Thus, the findings indicated the importance of tRNA modifications for the survival of *T. thermophilus*.

6. Utilization of tRNA Modification Enzymes from Thermophiles

Given that proteins from thermophiles are heat-resistant and very stable, numerous tRNA modification enzymes have been used in biochemical and structural studies (Tables 1 and 2). In particular, crystal structural studies of thermostable enzymes provided significant information on catalytic mechanisms and RNA-protein interactions. Studies on the crystal structures of tRNA modification enzymes from thermophiles are summarized in Supplementary Table S2. It is anticipated that thermostable proteins will continue to contribute structural studies in the future. Thermostable tRNA modification enzymes can be a tool for molecular and cell biology. For example, *A. fulgidus* TiaS with agmatine analogues has been used for site-specific RNA-labeling in mammalian cells [315]. In addition, thermostable tRNA modification enzymes may be used for healthcare. For example, Gm₁₈ modification in tRNA does not stimulate the Toll-like receptor 7 [287,288] and tRNA with Gm₁₈ alleviates inflammation [288]. Since TrmH from *T. thermophilus* can methylate all tRNA species [72] and is very stable, it may be useful for preparing tRNAs with Gm₁₈ modifications for tRNA therapy.

7. Perspective

Given that the temperature of ancient Earth was very high relative to that of present-day Earth, thermophiles may be remnants of ancient living organisms. Therefore, studies on tRNA modification enzymes and modified nucleosides in tRNA from thermophiles will contribute to the considerations of the evolutionary pathways of living organisms. Furthermore, such studies will continue to shed light on the variety and environmental adaptations of living organisms. Moreover, as outlined above, the thermostable enzymes may be useful as biotechnological and medical tools and may contribute toward the production of valuable materials.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-2607/6/4/110/s1>, Table S1: Abbreviations of modified nucleosides, Table S2: Crystal structural studies on tRNA modification enzymes from thermophiles.

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