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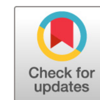

Distinct Modified Nucleosides in tRNA^{Trp} from the Hyperthermophilic Archaeon *Thermococcus kodakarensis* and Requirement of tRNA m^{2}G10/m$^2_2$G10 Methyltransferase (Archaeal Trm11) for Survival at High Temperatures

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ABSTRACT tRNA m^{2}G10/m$^2_2$G10 methyltransferase (archaeal Trm11) methylates the 2-amino group in guanosine at position 10 in tRNA and forms *N*^{2},*N*^{2}-dimethylguanosine (m$^2_2$G10) via *N*^{2}-methylguanosine (m^{2}G10). We determined the complete sequence of tRNA^{Trp}, one of the substrate tRNAs for archaeal Trm11 from *Thermococcus kodakarensis*, a hyperthermophilic archaeon. Liquid chromatography/mass spectrometry following enzymatic digestion of tRNA^{Trp} identified 15 types of modified nucleoside at 21 positions. Several modifications were found at novel positions in tRNA, including 2′-*O*-methylcytidine at position 6, 2-thiocytidine at position 17, 2′-*O*-methyluridine at position 20, 5,2′-*O*-dimethylcytidine at position 32, and 2′-*O*-methylguanosine at position 42. Furthermore, methylwyosine was found at position 37 in this tRNA^{Trp}, although 1-methylguanosine is generally found at this location in tRNA^{Trp} from other archaea. We constructed *trm11* (Δ*trm11*) and some gene disruptant strains and compared their tRNA^{Trp} with that of the wild-type strain, which confirmed the absence of m$^2_2$G10 and other corresponding modifications, respectively. The lack of 2-methylguanosine (m^{2}G) at position 67 in the *trm11 trm14* double disruptant strain suggested that this methylation is mediated by Trm14, which was previously identified as an m^{2}G6 methyltransferase. The Δ*trm11* strain grew poorly at 95°C, indicating that archaeal Trm11 is required for *T. kodakarensis* survival at high temperatures. The m$^2_2$G10 modification might have effects on stabilization of tRNA and/or correct folding of tRNA at the high temperatures. Collectively, these results provide new clues to the function of modifications and the substrate specificities of modification enzymes in archaeal tRNA, enabling us to propose a strategy for tRNA stabilization of this archaeon at high temperatures.

IMPORTANCE *Thermococcus kodakarensis* is a hyperthermophilic archaeon that can grow at 60 to 100°C. The sequence of tRNA^{Trp} from this archaeon was determined by liquid chromatography/mass spectrometry. Fifteen types of modified nucleoside were observed at 21 positions, including 5 modifications at novel positions; in addition, methylwyosine at position 37 was newly observed in an archaeal tRNA^{Trp}. The construction of *trm11* (Δ*trm11*) and other gene disruptant strains confirmed the enzymes responsible for modifications in this tRNA. The lack of 2-methylguanosine (m^{2}G) at position 67 in the *trm11 trm14* double disruptant strain suggested that this position is methylated by Trm14, which was previously identified as an m^{2}G6 methyltransferase. The Δ*trm11* strain grew poorly at 95°C, indicating that archaeal Trm11 is required for *T. kodakarensis* survival at high temperatures.

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Adaptor molecule tRNA is required for the conversion of genetic information encoded by nucleic acids to amino acid sequences in proteins. Numerous tRNA modifications are needed for sufficient and correct protein synthesis. To date, more than 100 modified nucleosides have been found in tRNAs from various living organisms (1). In particular, tRNAs from hyperthermophiles contain various modified nucleosides (2–6), which are thought to maintain the functions of tRNA at high temperatures. However, there are only a few examples of a tRNA sequence containing modified nucleosides from hyperthermophilic archaea (i.e., *Sulfolobus acidocaldarius* initiator $tRNA^{Met}$ [7] and, as published during the preparation of this report, *Methanocaldococcus jannaschii* tRNAs with several modifications mainly found in anticodon-arms in tRNAs [8]). In general, determining the sequence of tRNA from thermophiles is not so easy, because these tRNAs are structurally very rigid and contain numerous modified nucleosides. In some cases, preparation of standard compounds of modified nucleosides is necessary.

In a recent study, we reported the crystal structure of tRNA m^2G10/m^2_2G10 methyltransferase from *Thermococcus kodakarensis* (9), a hyperthermophilic archaeon that grows at 60 to 100°C (10). Archaeal tRNA m^2G10/m^2_2G10 methyltransferase catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to the 2-amino group in guanosine at position 10 (G10) in tRNA and forms N^2,N^2-dimethylguanosine (m^2_2G) via the intermediate N^2-methylguanosine (m^2G) (11). Although its eukaryotic counterpart (Trm11) requires another subunit (Trm112) (12, 13) for enzymatic activity (14), the archaeal enzyme does not require a partner subunit (11). Furthermore, the eukaryotic Trm11–Trm112 complex catalyzes a single methyl transfer reaction and forms only m^2G10 in tRNA. Therefore, the archaeal enzyme has been called Trm-G10 (11) or Trm-m^2_2G10 (15) to distinguish it from the eukaryotic enzyme. In this study, however, we use the name "archaeal Trm11" instead of Trm-G10 or Trm-m^2_2G10 enzyme, owing to the amino acid sequence similarity between the eukaryotic and archaeal enzymes (9).

Many types of modified nucleoside are specifically formed in individual tRNAs, and they are considered to confer various functional hallmarks on tRNA in a coordinated manner. To gain insight into the molecular and physiological roles of m^2_2G10 and Trm11, it is necessary to reveal the complete sequence of substrate tRNAs for Trm11, including other modified nucleosides. In the present work, we therefore determined the complete sequence of $tRNA^{Trp}$ isolated from *T. kodakarensis* and found several modified nucleosides at novel positions that have not been detected in any tRNA reported so far. Furthermore, established genetic manipulation systems for *T. kodakarensis* (16–20) enabled us to construct a Tk0981 (*trm11*) gene disruptant strain (Δ*trm11*) and additional gene disruptant strains responsible for other modified nucleosides. By analyzing $tRNA^{Trp}$ from the disruptant strains, we observed the lack of m^2_2G10 in $tRNA^{Trp}$ from the Δ*trm11* strain and confirmed that corresponding modified nucleosides were absent in individual gene disruptant strains.

We also studied the growth of the *trm11* gene disruptant (Δ*trm11*) strain at high temperatures. We discuss our findings in terms of the stability of tRNA in hyperthermophilic archaea and the survival of these microbes at high temperatures.

RESULTS

Purification and sequencing of $tRNA^{Trp}$ from *T. kodakarensis*. To determine all modified nucleosides formed in a substrate tRNA for Trm11 of *T. kodakarensis*, we used $tRNA^{Trp}$ as the target tRNA for the following reasons. First, there is only one $tRNA^{Trp}$ gene in the genome; therefore, the gene is causally expressed in *T. kodakarensis* cells. Second, the sequence of $tRNA^{Trp}$ differs considerably from that of other tRNA; therefore, it should be purified relatively easily by the solid-phase DNA probe method (21). Third, given that the

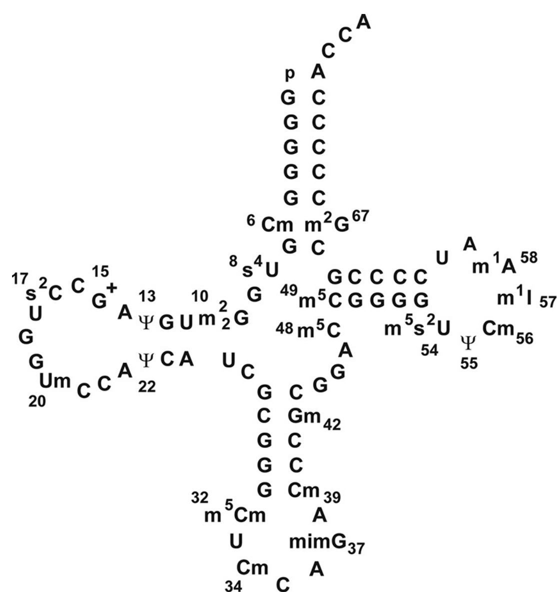

FIG 1 Cloverleaf structure of tRNA^{Trp} from *T. kodakarensis*. The modified nucleosides are defined in Table 1.

nucleosides at positions 6 and 26 in tRNA^{Trp} are both C (Fig. 1), it was expected that this tRNA would not be methylated by Trm14 (tRNA m^{2}G6 methyltransferase) (22) or Trm1 (tRNA m^{2}G26/m$^2_2$G26 methyltransferase) (20, 23–25) at the outset of the study. (As described below, we found that Trm14 can methylate a novel residue, G67, in this study.) Fourth, in our previous study, Trm11 of *T. kodakarensis* was revealed to methylate G at position 10 to m$^2_2$G by using *in vitro* transcribed tRNA^{Trp} (9), suggesting that cellular tRNA^{Trp} is one of the substrates for Trm11 *in vivo*. We successfully purified tRNA^{Trp} by a solid-phase DNA probe method.

The determined nucleoside sequence of tRNA^{Trp} is shown by a cloverleaf structure in Fig. 1 with positions numbered in accordance with the system described in reference 26. The modified nucleosides are defined in Table 1, and their structures are available from the Modomics database (http://modomics.genesilico.pl/) (1). The enzymes predicted to be responsible for the modified nucleosides, together with their genes, are given in Table 2.

The sequence shown in Fig. 1 was determined by liquid chromatography-mass spectrometry (LC/MS) analysis of digested tRNA^{Trp} from the wild-type strain. The base peak chromatograms of tRNA^{Trp} fragments derived from digestion with RNase T$_1$ and RNase A are shown in Fig. S1A and B, respectively, in the supplemental material. The nucleoside composition of each fragment was determined by comparing the measured *m/z* with the *m/z* calculated from the primary sequence of tRNA^{Trp} with possible modifications (Tables 3 and 4). The sequences of the fragments and modification sites were assigned by collision-induced dissociation (CID) (Fig. S1C). Pseudouridine (Ψ), a mass-silent uridine modification, was identified in a similar way, but with derivatization to 1-cyanoethyl Ψ by acrylonitrile treatment prior to RNase digestion (Fig. S1C). In these analyses, Cm32 was found to be further methylated (RNase A-derived fragment 4). We deduced that the second methylation would be a base methylation: m^{5}Cm has been found specifically in thermophilic archaea (2, 4–6). In humans, the *ALKBH1* gene is responsible for f^{5}Cm34 formation in tRNA$^{Leu}_{CAA}$ (27): in *ALKBH1* knockout cells, the intermediate m^{5}Cm34 is found in tRNA$^{Leu}_{CAA}$ instead of the final product (f^{5}Cm34). Here, therefore, we used this modified nucleoside (m^{5}Cm) as a standard marker. We purified tRNA$^{Leu}_{CAA}$ from human *ALKBH1* knockout cells and tRNA^{Trp} from *T. kodakarensis* and digested them to nucleosides, which were then mixed and analyzed by LC/MS (Fig. 2). The dimethylated C in *T. kodakarensis* tRNA^{Trp} was eluted at the same time as the

TABLE 1 Abbreviations of modified nucleosides used in this study

Abbreviation	Modified nucleoside
m^3C	3-Methylcytidine
m^4C	*N*[4]-Methylcytidine
f^5Cm	5-Formyl-2′-*O*-methylcytidine
D	Dihydrouridine
m^5U	5-Methyluridine
m^1G	1-Methylguanosine
m^7G	7-Methylguanosine
Ψm	2′-*O*-Methylpseudouridine
m^1Im	1,2′-*O*-Dimethylinosine
$m^2{}_2Gm$	N^2,N^2,2′-*O*-Trimethylguanosine
s^2U	2-Thiouridine
ac^6A	N^6-Acetyladenosine
Cm	2′-*O*-Methylcytidine
s^4U	4-Thiouridine
$m^2{}_2G$	N^2,N^2-Dimethylguanosine
Ψ	Pseudouridine
G^+	Archaeosine
s^2C	2-Thiocytidine
Um	2′-*O*-Methyluridine
m^5Cm	5,2′-*O*-Dimethylcytidine
mimG	Methylwyosine
Gm	2′-*O*-Methylguanosine
m^5C	5-Methylcytidine
m^5s^2U	5-Methyl-2-thiouridine
m^1I	1-Methylinosine
m^1A	1-Methyladenosine
m^2G	N^2-Methylguanosine
imG-14	4-Demethylwyosine
imG2	Isowyosine
imG	Wyosine
yW-86	7-Aminocarboxypropyldemethylwyosine
yW-72	7-Aminocarboxypropylwyosine
Am	2′-*O*-Methyladenosine

standard m^5Cm by LC (Fig. 2, top), and CID analysis showed that the cytosine base is monomethylated (Fig. 2, bottom). On the basis of these results, we concluded that a portion of Cm32 is modified to m^5Cm32 in $tRNA^{Trp}$. All modifications were also confirmed by LC/MS analysis of nucleosides derived from complete digestion

TABLE 2 Predicted enzymes and genes for $tRNA^{Trp}$ nucleoside modifications[a]

Modified nucleoside and position	Enzyme(s) or RNA	Predicted gene ID
Cm6	Unknown	Unknown
s^4U8	ThiI	Tk0366
$m^2{}_2G10$	Archaeal Trm11	Tk0981
Ψ13	TruD	Tk2302
G^+15	ArcTGT, ArcS	Tk0760, Tk2156
s^2C17	Unknown	Unknown
Um20	L7Ae, Nop5, archaeal fibrillarin, C/D-box guide RNA	Tk1311, Tk0184, Tk0183, RNA
Ψ22	Unknown	Unknown
m^5Cm32	Unknown methyltransferase, archaeal TrmJ	Unknown, Tk1970
Cm34	L7Ae, Nop5, archaeal fibrillarin, intron (C/D-box guide RNA)	Tk1311, Tk0184, Tk0183, intron
mimG37	Trm5b, TYW1, TYW3, Trm5a	Tk0497, Tk1671, Tk0175, Tk2223
Cm39	L7Ae, Nop5, archaeal fibrillarin, intron (C/D-box guide RNA)	Tk1311, Tk0184, Tk0183, intron
Gm42	L7Ae, Nop5, archaeal fibrillarin, C/D-box guide RNA	Tk1311, Tk0184, Tk0183, RNA
m^5C48	Archaeal Trm4	Tk0360
m^5C49	Archaeal Trm4	Tk0360
m^5s^2U54	RumA, TtuA?, TtuB?, α	Tk2134, Tk1556?, Tk1093?, α
Ψ55	Pus10 or archaeal Cbf5	Tk0903 or Tk1509
Cm56	Trm56	Tk0060
m^1I57	Archaeal TrmI, unknown deaminase	Tk1328, unknown
m^1A58	Archaeal TrmI	Tk1328
m^2G67	Trm14	Tk1863

[a]?, enzymatic activity of the protein has not been confirmed in archaea.

TABLE 3 List of fragments of *T. kodakarensis* tRNA^{Trp} after digestion with RNase T$_1$[a]

			Monoisotopic *m/z*		
Fragment no.	**Fragment sequence**	**Mol wt**	**Calculated**	**Observed**	**Charge state**
1	CmUCmCAmimGACmCCGmCGp	4,274.682	711.439	711.439	−6
2	m^{5}s^{2}UΨCmm^{1}Im^{1}AAUCCCCGp	3,908.532	643.412	643.414	−6
3	UmCCAΨCAUCGp	3,173.420	633.676	633.676	−5
3′	UCCAΨCAUCGp	3,159.404	630.873	630.874	−5
4	CCCCCACCAOH (3′ terminal)	2,731.438	1,363.711	1,364.713	−2
5	ΨAG$^+$Cs^{2}CUGp	2,316.295	1,157.134	1,157.139	−2
5′	ΨAG$^+$CCUGp	2,300.318	1,149.151	1,149.149	−2
6	Am^{5}Cm^{5}CGp	1,330.224	664.104	664.103	−2
6′	ACm^{5}CGp	1,316.209	657.097	657.099	−2
7	m$^2_2$GUGp	1,042.162	1,041.154	1,041.152	−1
8	s^{4}UGp	685.060	684.053	684.051	−1
9	Cm^{2}Gp, CmGp	682.115	681.107	681.106	−1
8′	UGp	669.083	668.075	668.074	−1
10	CGp	668.099	667.091	667.089	−1

[a]Partially modified fragments detected in reasonable quantity are indicated. "OH" and "p" indicate the 3′ terminal hydroxyl group and terminal phosphate, respectively.

of tRNA^{Trp} (Fig. S2). All of the fragments detected with modifications are listed in Tables 3 and 4.

m$^2_2$G10 formation by Trm11 *in vivo* and growth phenotype of the *trm11* gene disruption. In the wild-type tRNA^{Trp}, m$^2_2$G was detected in RNase T$_1$-derived fragment 7 and RNase A-derived fragment 8, indicating that m$^2_2$G is present at position 10. No m^{2}G at position 10, an intermediate of m$^2_2$G10, was detected in our analysis (data not shown), indicating that m$^2_2$G10 is efficiently introduced by Trm11 *in vivo*. To confirm that the *trm11* gene is responsible for the m$^2_2$G10 modification, we constructed a *trm11* gene disruptant (Δ*trm11*) strain (Fig. S3 and S4). The methods for construction of the gene disruptant strain are described in the supplemental material. The m$^2_2$G nucleoside was not detected in the corresponding fragments of tRNA^{Trp} from the Δ*trm11* strain (Fig. S5), and the nucleoside at position 10 was confirmed as unmodified G. We therefore concluded that the *trm11* gene is responsible for the m$^2_2$G10 modification in tRNA^{Trp}. During the preparation of this paper, it was reported that the m$^2_2$G content in total tRNA from a *T. kodakarensis trm11* gene disruptant strain, which was obtained by

TABLE 4 List of fragments of *T. kodakarensis* tRNA^{Trp} after digestion with RNase A[a]

			Monoisotopic *m/z*		
Fragment no.	**Fragment sequence**	**Mol wt**	**Calculated**	**Observed**	**Charge state**
1	pGGGGGCmGs^{4}Up (5′ terminal)	2,809.321	1,403.653	1,403.651	−2
1′	pGGGGGCmGUp (5′ terminal)	2,793.344	1,395.664	1,395.665	−2
2	GGGGm^{5}s^{2}UΨp	2,040.244	1,019.114	1,019.115	−2
3	AmimGACmCp	1,711.308	854.646	854.650	−2
4	GGGm^{5}CmUp	1,692.251	845.118	845.114	−2
4′	GGGCmUp	1,678.235	838.110	838.114	−2
5	Cmm^{1}Im^{1}AAUp	1,659.266	828.625	828.627	−2
6	GGAm^{5}Cp	1,356.215	677.100	677.097	−2
6′	GGACp	1,342.199	670.092	670.095	−2
7	GGUmCp	1,333.188	665.586	665.588	−2
8	Gm$^2_2$GUp	1,042.162	1,041.154	1,041.155	−1
9	AG$^+$Cp	1,038.178	1,037.171	1,037.170	−1
7′	GGUp	1,014.131	1,013.123	1,013.124	−1
10	GmCp	682.115	681.107	681.107	−1
11	m^{2}GCp	682.115	681.107	681.106	−1
12	GΨp	669.083	668.075	668.075	−1
13	GCp	668.099	667.091	667.091	−1
14	AUp, AΨp	653.088	652.081	652.081	−1
15	ACp	652.104	651.097	651.097	−1
16	CmCp	642.109	641.101	641.102	−1

[a]Partially modified fragments detected in reasonable quantity are indicated. "OH" and "p" indicate the 3′ terminal hydroxyl group and terminal phosphate, respectively.

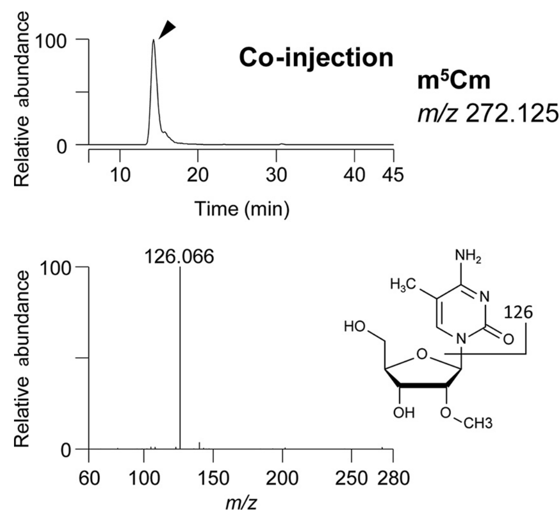

FIG 2 Position 32 is modified to m^{5}Cm in *T. kodakarensis* tRNA^{Trp}. Top, extracted ion chromatography (XIC) showing coelution of the nucleoside modified at position 32 in tRNA^{Trp} from *T. kodakaraensis* and m^{5}Cm in human cytoplasmic tRNA$^{Leu}{}_{CAA}$ from *ALKBH1* knockout cells. Bottom, CID spectrum of m^{5}Cm. The cleavage position of the base-related ion is indicated on the chemical structures.

transposon random mutagenesis, was decreased relative to that from the wild-type strain (28). Our results provide experimental support for that observation.

We hypothesized that the m$^2{}_2$G10 modification might be required for the survival of *T. kodakarensis* at high temperatures. We therefore measured the growth of the Δ*trm11* strain at 85, 90, 93 and 95°C. In addition, we constructed a complemented (Δ*trm11* + *trm11*) strain to confirm that the growth phenotype observed was due to the lack of Trm11. The *trm11* gene was reinserted into the *chiA* (Tk1765; chitinase gene) region in the genomic DNA of the Δ*trm11* strain. Deletion of the Tk1765 gene does not cause growth defects unless chitin is used as a carbon source (29). Although its expression level was lower in the complemented strain than in the wild-type strain, Trm11 was expressed in the complemented strain, as determined by Western blotting (Fig. 3A). At 85°C, the wild-type, Δ*trm11*, and complemented strains showed similar growth curves (Fig. 3B). As the temperature increased, however, the growth of the Δ*trm11* strain was clearly slower than that of the wild-type or complemented strain. At 95°C, the Δ*trm11* strain showed a considerable growth defect, whereas the complemented strain grew at approximately the same speed as the wild-type strain, indicating that the growth defect of the Δ*trm11* strain is due to the lack of archaeal Trm11 protein. The study based on random mutagenesis reported that the *trm11* gene product is required for the effective growth of *T. kodakarensis* at 93°C (28). Although there is a slight difference in the growth speeds between our data and those data at 93°C, this might be due to differences in the culture conditions. Collectively, these observations reveal that Trm11 is required for the survival of *T. kodakarensis* at high temperatures.

Validation of predicted *thiI*, *rumA*, and *TYW1* genes. In general, s^{4}U8 modification in eubacterial and archaeal tRNA is performed by ThiI (30). To determine whether the s^{4}U8 modification in tRNA^{Trp} is carried out by ThiI, we analyzed tRNA^{Trp} from the Δ*thiI* strain. Whereas RNase A-derived fragment 1 (pGGGGCmGs^{4}Up) was clearly detected in the wild-type sample (Fig. 4A, left), this fragment was not found in the Δ*thiI* sample and only RNase A-derived fragment 1′ (pGGGGCmGUp) was detected (Fig. 4A, right). These results confirm that the s^{4}U8 modification in tRNA^{Trp} is conferred by ThiI.

S-Adenosyl-L-methionine-dependent tRNA m^{5}U54 methyltransferase activity was previously detected in the cell extract of *Pyrococcus furiosus* (31), and the responsible *rumA*-like gene was identified from *Pyrococcus abyssi* and *T. kodakarensis* (32). To determine whether the *rumA* gene (Tk2134) is responsible for the 5-methylation of U54 in *T. kodakarensis*, we analyzed tRNA^{Trp} from the Δ*rumA* strain. RNase T$_1$-derived

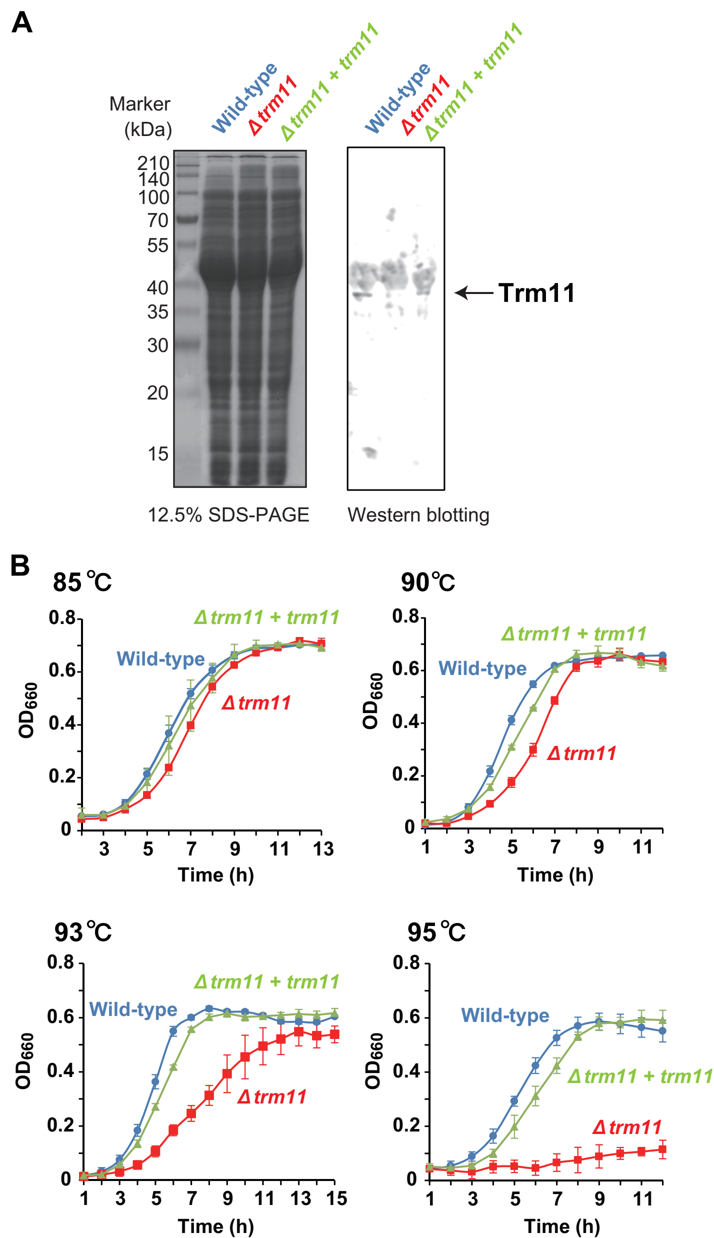

FIG 3 The *trm11* gene disruptant strain shows defective growth at high temperature. (A) Western blot confirming the expression of Trm11 protein in the complemented (Δ*trm11* + *trm11*) strain. Left, proteins in the cell extracts from wild-type, Δ*trm11*, and complemented strains were separated by 12.5% SDS-PAGE. The gel was stained with Coomassie brilliant blue. Right, proteins were transferred to a membrane, and Western blotting was performed. (B) Growth of the wild-type, Δ*trm11*, and complemented (Δ*trm11* + *trm11*) strains was measured at 85, 90, 93, and 95°C. Error bars indicate the standard deviations of results of three independent culture experiments.

fragment 2 (GGGGm^{5}s^{2}UΨp) was detected in the wild-type sample (Fig. 4B, left) but not in the Δ*rumA* sample, which instead contained a new fragment (GGGGs^{2}UΨp) (Fig. 4B, right, and C). This finding indicated that the *rumA* gene is responsible for the m^{5}U54 modification and also that s^{2}U54 formation is not dependent on the presence of a 5-methyl group in m^{5}U54.

The mimG (33) nucleoside is one of the final products of the biosynthetic pathway of archaeal wyosine derivatives (Fig. 5A) (34, 35). Traditionally, mimG was thought to exist only in tRNA^{Phe}. Recently, however, it was reported that imG-14 and imG are present at position 37 in several tRNAs from *M. jannaschii* (8). In that study, the modified

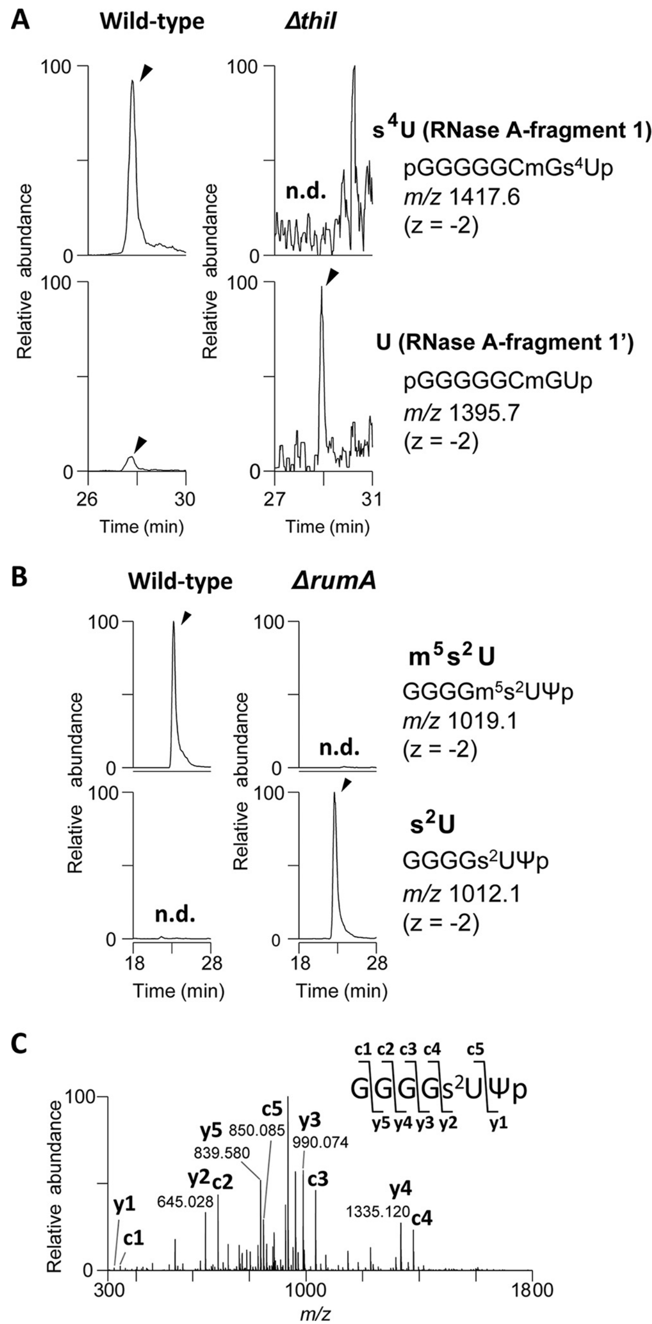

FIG 4 The *thiI* and *rumA* genes are responsible for the formation of s^4U8 and 5-methylation of U54, respectively, in $tRNA^{Trp}$. (A) XICs of RNase A-digested fragments containing s^4U (top) or U (bottom) at position 8 (arrowheads) are shown. The sequences, *m/z*, and charge states are indicated on the right. n.d., not detected. (B) XICs of an RNase A-digested fragment containing m^5s^2U (top) or s^2U (bottom) at position 54 (arrowheads). The sequence, *m/z*, values, and charge states are indicated on the right. (C) CID spectrum of the RNase A-derived fragment from the Δ*rumA* strain. The sequence and assigned signals are shown in the inset (precursor, doubly charged ions of *m/z* 1,012.1).

nucleoside at position 37 in $tRNA^{Trp}$ from *M. jannaschii* was determined to be m^1G37 (8). In our study, however, LC/MS analysis indicated the presence of a modified nucleoside corresponding to mimG (*m/z* 350.146) at position 37 of $tRNA^{Trp}$. To confirm the presence of mimG in $tRNA^{Trp}$, we analyzed $tRNA^{Trp}$ from a Δ*TYW1* strain in which the gene encoding TYW1, the enzyme catalyzing the second step of mimG synthesis, was disrupted. We considered that if the modified nucleoside at position 37 is mimG, then m^1G37, the first product of the mimG synthesis pathway catalyzed by archaeal

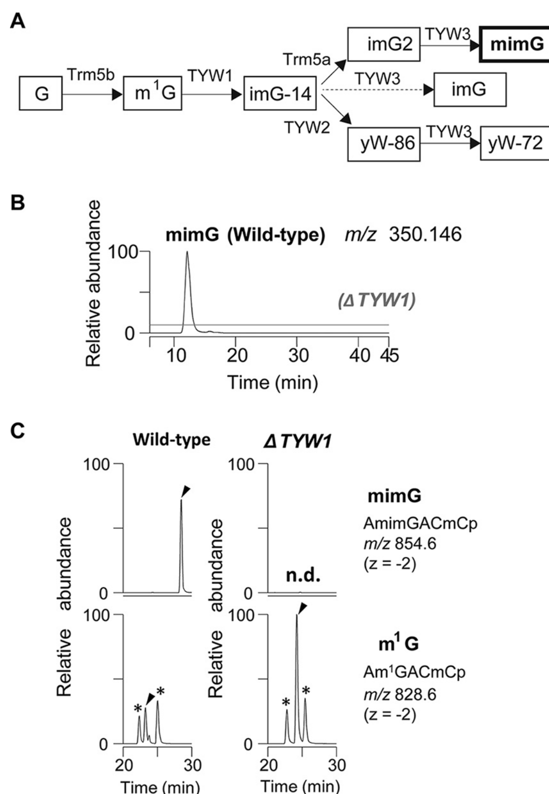

FIG 5 Methylwyosine is present at position 37 in tRNA^{Trp}. (A) Predicted biosynthetic pathway of wyosine derivatives in *T. kodakarensis*. This figure is based on data from a report by de Crécy-Lagard et al. (34). The abbreviations of modified nucleotides are listed in Table 1. The predicted enzymes are indicated. (B) Nucleoside analysis of tRNA^{Trp} from wild-type and Δ*TYW1* strains. mimG is not observed in the Δ*TYW1* sample. (C) In the RNase A fragment from the Δ*TYW1* strain, m^{1}G is observed at position 37 instead of mimG37. Asterisks show other eluates with almost the same *m/z* values. n.d., not detected.

Trm5b (Fig. 5A) (36–38), should be detected in tRNA^{Trp} from the Δ*TYW1* strain. As expected, the modified nucleoside corresponding to mimG (*m/z* 350.146) was not observed in the nucleosides from the digested tRNA^{Trp} from the Δ*TYW1* strain (Fig. 5B). Furthermore, RNase A-derived fragment 3 (AmimGACmCp) disappeared and a new RNase A-derived fragment (Am^{1}GACmCp) appeared (Fig. 5C). Taking the results altogether, we concluded that mimG37 is present in tRNA^{Trp} from *T. kodakarensis*.

The *trm14* gene is responsible for the m^{2}G67 modification. The m^{2}G67 modification was previously found in tRNA^{Lys} from *Loligo bleekeri* (39). Furthermore, it has been reported that tRNA^{Arg}, tRNA^{Asn}, tRNA^{Gly}, tRNA^{Ile}, and tRNA^{Val} from *M. jannaschii* contain m^{2}G67 (8). The modification site (G67) forms a Watson-Crick base pair with C6 in tRNA. Archaeal Trm14 methylates G6 in tRNA and contains a THUMP domain (22, 40), which often recognizes the CCA terminus in tRNA (9, 41, 42). Therefore, we considered that Trm14 may be responsible for the m^{2}G67 modification in tRNA^{Trp}. To test this idea, we analyzed tRNA^{Trp} from the *trm11 trm14* double disruptant (Δ*trm11* Δ*trm14*) strain (Fig. S6): the construction of the Δ*trm11* Δ*trm14* strain is described in the supplemental text. As shown in Fig. 6, the m^{2}G nucleoside (Fig. 6A) and the RNA fragment (m^{2}GCp) (Fig. 6B) completely disappeared in the sample from the Δ*trm11* Δ*trm14* strain, demonstrating that the Trm14 is responsible for the m^{2}G67 modification in tRNA.

DISCUSSION

Our present study revealed the complete sequence of tRNA^{Trp} from *T. kodakarensis* as the first instance of this species. The result that 15 modified nucleosides were found at 21 positions provides insight into their molecular function and their modifying genes or enzymes. Indeed, we successfully confirmed that *trm11* is the gene responsible for

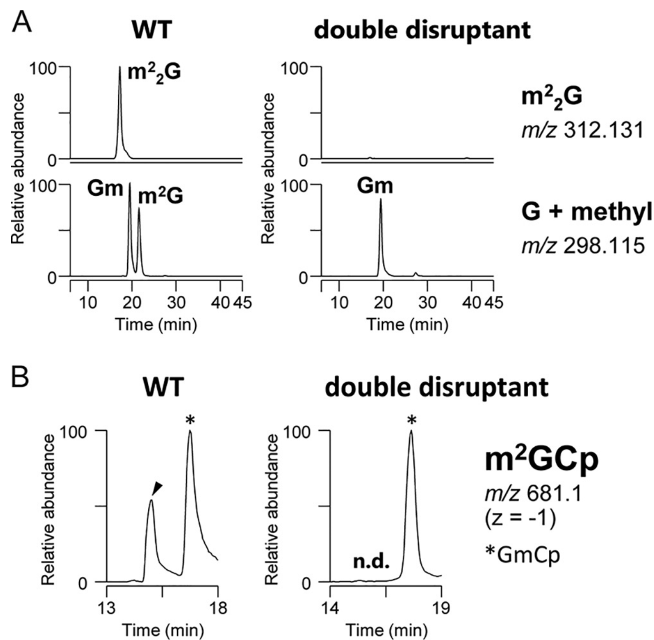

FIG 6 The *trm*14 gene is responsible for m^2G67 formation. (A) Nucleoside analysis of $tRNA^{Trp}$ from wild-type (WT) and Δ*trm11* Δ*trm14* (double disruptant) strains. $m^2{}_2G$ and m^2G are absent in the double disruptant strain. (B) XICs tracing an RNase A-digested fragment containing m^2G at position 67 (arrowhead). The sequences, *m/z*, and charge states are indicated on the right. Asterisks show GmCp (Table 4) with the same *m/z* value as the m^2GCp fragment. n.d.; not detected.

$m^2{}_2G$ at position 10 as well as *thiI* for s^4U, *rumA* for m^5U, *TYW1* for imG-14, and *trm14* for m^2G at positions 8, 54, 37, and 67, respectively, by analysis of $tRNA^{Trp}$ from gene disruptant strains. Notably, the requirement of *trm14* for m^2G67 formation has not previously been reported. The functional features and biogenesis of modified nucleosides in the $tRNA^{Trp}$ are discussed below in detail.

To our knowledge, Cm6 has not previously been found in tRNAs from archaea, eubacteria, and eukaryotes. However, Am6 formation activity was previously detected in the cell extract of *Pyrococcus furiosus* (24). Therefore, a novel tRNA 2′-*O*-methyltransferase, which methylates the 2′-OH of ribose at position 6 in tRNA and does not differentiate between adenine and cytosine, may exist in *Thermococcus* and *Pyrococcus* genera. In terms of the other enzymes responsible for the observed 2′-*O*-methylations, Cm56 is a product of Trm56 (43, 44). The Um20 and Gm42 modifications are likely to be products of L7Ae, Nop5, archaeal fibrillarin (aFib), and the C/D-box guide RNA system (45, 46), with the following predicted C/D-box RNAs: 5′-CCU G<u>AU GAU GA</u>G UAA ACC CGU UG<u>C UGA</u> GAA AAA G<u>AU GAU GA</u>U GGA UGG ACC AG<u>C UGA</u> CC-3′ (coding region, positions 159454 to 159512) for Um20, and 5′-CGG G<u>AU GAU GA</u>G UCU GGA GCC CC<u>C UGA</u> GAG <u>GUG AAG A</u>GG UUU CGC GGG G<u>CU GA</u>C C-3′ (coding region, positions 1371729 to 1371783) for Gm42 (underlining indicates the sequences of the C, D′, C′, and D boxes). Furthermore, Cm34 and Cm39 are also products of L7Ae, Nop5, aFib, and the C/D-box guide RNA system. In this case, an intron in precursor $tRNA^{Trp}$ functions as the guide RNA (47–49). Notably, the gene of *T. kodakarensis* $tRNA^{Trp}$ contains a similar intron (50). 2′-*O*-Methylated nucleosides at multiple positions in tRNA can stabilize the tRNA structure (6). For example, *Pyrodictium occultum* can grow at 105°C, and various 2′-*O*-methylated nucleosides such as 2′-O-methylpseudouridine (Ψm), 1,2′-O-dimethylinosine (m^1Im), and *N*²,*N*²,2′-*O*-trimethylguanosine ($m^2{}_2Gm$) are present in tRNA from this archaeon: however, 2-thiouridine (s^2U) and 5-methyl-2-thiouridine (m^5s^2U) are not found (2, 51). Whereas the melting temperature of the *P. occultum* $tRNA^{Met}$ transcript is 80°C, that of the native $tRNA^{Met}$ is more than 100°C (52), indicating that the melting temperature of *P. occultum* tRNA is increased by more than 20°C via a combination of numerous 2′-*O*-methylated nucleosides. In general, 2′-*O*-

methylation shifts the equilibrium of ribose puckering to the C3′-endo form and enhances the hydrophobic interaction. Thus, 2′-*O*-methylation is one of the strategies to maintain tRNA structure at high temperatures.

The m^{5}Cm modification has been considered to be specific to thermophilic archaea (2, 4–6). So far, the only exception in mesophiles is the intermediate of f^{5}Cm34 synthesis observed in human tRNA$^{Leu}{}_{CAA}$. For a long time, however, the position of m^{5}Cm in tRNA from thermophilic archaea has remained unclear, and, to our knowledge, our study is the first to clarify the presence of m^{5}Cm at position 32 in tRNA from these microbes. The 2′-*O*-methylation of m^{5}Cm32 is probably performed by archaeal TrmJ. The substrate RNA specificity of *S. acidocaldarius* TrmJ was previously investigated using several mutant tRNA transcripts (53); that study suggests that methylation of ribose of C32 in *T. kodakarensis* tRNA^{Trp} can occur after removal of the intron. It has been reported that C32 in tRNA^{Trp} from *M. jannaschii* is modified to s^{2}C32 (8); the modification pathway of C32 in tRNA^{Trp} differs between *T. kodakarensis* and *M. jannaschii*. The Cm32 modification is often observed in tRNAs that are used to decode codons in one- and two-codon boxes (54). In terms of archaeal tRNA m^{5}C methyltransferases, for a long time, only Trm4 had been characterized. In 1999, the enzymatic activity of Trm4 was detected in the cell extract of *P. furiosus* as tRNA m^{5}C49 methyltransferase (31). Subsequently, it was found that Trm4 changes its methylation site in the presence of archease (55). Archaeal Trm4 is now known to be a multiple-site-specific tRNA methyltransferase for m^{5}C48 and m^{5}C49 modifications (55). Therefore, m^{5}C48 and m^{5}C49 modifications in *T. kodakarensis* tRNA^{Trp} can be explained by the enzymatic activity of Trm4. Furthermore, during the preparation of this paper, it was reported that *Pyrococcus horikoshii* NSUN6 methylates C72 and forms m^{5}C72 in several tRNAs (56). However, a tRNA m^{5}C32 methyltransferase has not been reported in any of the three domains of life. It is possible that the 5-methyl group in m^{5}C32 enhances the stacking effect of C32 with G31 and contributes to stabilizing the anticodon arm at high temperatures.

Three types of sulfur-containing modifications were present in tRNA^{Trp}. Although the s^{4}U modification has been observed in unfractionated tRNA nucleosides from several archaea (3), it has not been found in tRNAs from haloarchaea (57–59) or initiator tRNA^{Met} from *S. acidocaldarius* (7); therefore, the modified position(s) of s^{4}U in tRNA has been confirmed in limited tRNAs (position 8 in *Thermoplasma acidophilum* elongator tRNA^{Met} [60], *Methanosarcina barkeri* tRNA^{Pyr} [61], and several *M. jannaschii* tRNAs [8] and positions 8 and 9 in *T. acidophilum* tRNA^{Leu} [62]). In terms of enzymatic properties, ThiI from *Methanococcus maripaludis* has been recently shown to contain a 3Fe-4S cluster and use inorganic sulfur compounds as sulfur donors (63).

Although the 2-thiocytidine (s^{2}C) nucleoside has been observed in tRNAs from several archaea (2, 3, 5), position 17 represents a novel modification site of s^{2}C. In tRNAs from mesophiles, position 17 is often modified to D17 (64). In *Escherichia coli* and *Saccharomyces cerevisiae*, for example, U17 in tRNA is modified to D17 by DusB (65, 66) and Dus1 (67), respectively. Nuclear magnetic resonance (NMR) analyses have suggested that D may destabilize the structure of tRNA by promoting the C2′-endo form of ribose (68). In general, therefore, D is thought to enhance the flexibility of tRNA. In contrast, the 2-thio group in s^{2}C promotes the C3′-endo form of ribose. Thus, the conformation of the D-loop in *T. kodakarensis* tRNA^{Trp} seems to be different from that in tRNA from mesophiles. Possibly, s^{2}C17 is required to maintain the D-loop structure (and interaction of the T and D arms) at high temperatures. The s^{2}C modification is usually found at position 32 in eubacterial tRNAs (e.g., *Escherichia coli* tRNA^{Arg} [69]) and archaeal tRNA (8). In *E. coli* and *Salmonella enterica* serovar Typhimurium, the s^{2}C32 modification in tRNA is performed by TtcA (70), which possesses a 4Fe-4S cluster (71). A *ttcA*-like gene (Tk1821) is included in the *T. kodakarensis* genome (71); however, the biosynthetic pathway of s^{2}C17 is currently unknown.

It was shown that the biogenesis of m^{5}s^{2}U54 is mediated independently by a methyltransferase RumA and an unidentified 2-thiolation system. This feature is common to the formation of m^{5}s^{2}U54 in eubacterial tRNA (72, 73); however, whereas the

methylation step in the m^5s^2U54 formation of archaeal tRNA is conferred by the *S*-adenosyl-L-methionine-dependent enzyme (RumA) (32), that of eubacterial tRNA is conferred by a folate- and FAD-dependent enzyme (TrmFO) (74, 75). In *T. kodakarensis*, two proteins homologous to TtuA (Tk1556 gene product) and TtuB (Tk1093 gene product) may be involved in the 2-thiolation of m^5s^2U54, as in eubacteria (76). Given that archaea do not possess a homolog of the IscS protein (77), however, the complete 2-thiolation system for the formation of m^5s^2U54 in *T. kodakarensis* tRNA remains unknown. The 2-thiolation of U54 has been found only in tRNAs from thermophiles such as *Aquifex aeolicus* (78) and *Thermus thermophilus* (72, 79, 80). The m^5s^2U54 modification forms a reverse Hoogsteen base pair with A58 (or m^1A58) in tRNA, like m^5U54 and $m^1\Psi54$ (81), and the 2-thio group in m^5s^2U54 enhances the stacking effect with the G51-C61 base pair (80). Because the 2-thio modification at position 54 increases the melting temperature of tRNA by more than 3°C (72, 76, 79, 80, 82), the m^5s^2U54 modification probably contributes to stabilization of the tRNA structure even in the case of *T. kodakarensis*.

In this study, we found mimG37 in $tRNA^{Trp}$. Archaeal Trm5 can recognize the guanine base in an A36G37 sequence (83), and there are no reports of the tRNA specificity of TYW1, TYW2, and TYW3. Thus, our findings do not conflict with the results of previous studies. Because position 36 in *T. kodakarensis* $tRNA^{Trp}$ is an unmodified A nucleoside, mimG37 may contribute to stabilize the base pair between A36 and U in mRNA during the protein synthesis at high temperatures. This idea is consistent with the fact that a mesophilic archaeon, *Haloferax volcanii*, does not contain wyosine derivatives in tRNA (58, 84).

G^+15 (85) is formed by ArcTGT (86) and ArcS (87), and ArcTGT from *T. kodakarensis* modifies only G15 in tRNA (20). During the preparation of this paper, it was reported that an ArcTGT gene disruptant mutant of *T. kodakarensis* cannot grow at 93°C (28). The m^1I57 modification is produced by deamination of m^1A57, which is carried out by archaeal TrmI (88, 89). Although deaminase activity has been detected in the cell extract of *H. volcanii* (90), the responsible gene(s) has not been identified as yet. The m^1A58 modification is a product of archaeal TrmI (88), and a recent random mutation study revealed that the *trmI* gene disruption strain cannot grow at 93°C (28).

In this study, we confirmed that the gene responsible for m^2G67 is *trm14*. However, it has not been confirmed that *T. kodakarensis* Trm14 methylates G6 in tRNA like *M. jannaschii* Trm14. Furthermore, the presence of m^2G6 modification in *T. kodakarensis* tRNAs has not been confirmed. To clarify these issues, further investigation is necessary.

In *T. kodakarensis*, Trm10 has been reported to methylate both G9 and A9 in tRNA, forming m^1G9 and m^1A9, respectively (91). Our analysis found, however, that G9 in $tRNA^{Trp}$ was unmodified. Therefore, *T. kodakarensis* Trm10 seems to methylate specific tRNAs. Recently, kinetic analysis of *T. kodakarensis* Trm10 revealed that the rate-determining step for catalysis involves a conformational change of the substrate tRNA (92).

The $\Psi55$ modification in $tRNA^{Trp}$ is likely to be performed by archaeal Pus10 (93–95) or archaeal Cbf5 (95–98). Because *Sulfolobus solfaraticus* Pus7 has been reported to possess weak activity for $\Psi13$ formation (99), the homologous protein (annotated as TruD [100] in the database [54]; Tk2302 gene product) may form $\Psi13$ in $tRNA^{Trp}$ from *T. kodakarensis*. The enzyme responsible for $\Psi22$ formation in archaeal tRNA is unknown (101). The contribution of a $\Psi13$-$\Psi22$ base pair to tRNA structure has been recently reviewed (102): this base pair may stabilize the D-arm structure at high temperatures.

It is an intriguing finding that the lack of Trm11 impacted the viability of *T. kodakarensis* at high temperatures. Because m^2_2G does not form a Watson-Crick base pair with C, m^2_2G10 may contribute to the folding of specific tRNAs, such as $tRNA^{Pro}$ from *P. abyssi* (15). In the case of *T. thermophilus*, an extremely thermophilic eubacterium, tRNA modification enzymes and the modified nucleosides in tRNA form a network (6, 72, 73, 103–106). At high temperatures, three modified nucleosides, m^5s^2U54 (76), m^1A58 (107), and m^7G46 (103), are essential for the survival of *T. thermophilus*. As

described above, the 2-thio group in m^5s^2U54 increases the melting temperature of tRNA. The m^1A58 modification is known to be a positive determinant of the sulfur transfer system used for m^5s^2U54 formation (76). The presence of m^7G46 in tRNA increases the activity of several tRNA modification enzymes, such as TrmH (108, 109) for Gm18, TrmD (110) for m^1G37, and TrmI (105, 107) for m^1A58. It is possible that thermophilic archaea possess a similar network of tRNA modification enzymes and modified nucleosides in tRNA. Indeed, the requirement of the archaeal *trmI* gene (TrmI produces m^1A57 and m^1A58) for the m^5s^2U54 modification in *T. kodakarensis* has been reported previously (28). We attempted to measure the melting temperature of tRNA mixtures from the Δ*trm11* strain, but it was not possible to determine it accurately because it was above 100°C in the presence of 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 100 mM NaCl (data not shown). Therefore, the growth defect of the Δ*trm11* strain at 95°C cannot be explained simply by the melting temperature of tRNA. As predicted for tRNA from *P. abyssi*, the $m^2{}_2G10$ modification in tRNA from *T. kodakarensis* might have an effect on folding of a specific tRNA(s) at high temperatures. To clarify these issues, further studies are required.

MATERIALS AND METHODS

Strains, media, and culture conditions. The strains of *T. kodakarensis* used in this study are listed in Table S1 in the supplemental material. The culture methods for *T. kodakarensis* KUW1 (17), KUWA (20), and gene disruptant strains are described in the supplemental text.

Disruption of *trm11* (Tk0981), *trm14* (Tk1863), *rumA* (Tk2135), *thiI* (Tk0368), and *TYW1* (Tk1671) genes. The plasmids used for gene disruptions are listed in Table S1. The primers used for genetic manipulations are listed in Table S2. The constructions of gene disruptant strain are described in the supplemental text.

Construction of a complemented strain expressing Trm11 in the Δ*trm11* strain. The conditional expression system in *T. kodakarensis* has been previously described (29). The construction of a complemented strain is described in the supplemental text.

Western blotting. The recombinant 6×His-tagged Trm11 protein was prepared as described previously (9) and used to immunize rabbits and obtain antibodies (Eurofins Genomics, Inc., Japan). Cell extracts of the wild-type and Δ*trm11* strains were prepared from cells grown to an optical density at 660 nm (OD_{660}) of ~0.6. A 1-ml aliquot of cells was mixed with 10 μl of 2× SDS-PAGE loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 2.5% SDS, 0.2% bromophenol blue, and 20% glycerol), boiled for 5 min, and then applied to a 12.5% SDS-PAGE gel. The gel was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) in accordance with the manufacturer's instructions. Trm11 protein was detected by using Alexa Fluor 488–anti-rabbit IgG (Invitrogen) as a secondary antibody and visualized with a Typhoon FLA 7000 laser scanner (GE Healthcare). For the complemented strain (Δ*trm11* + *trm11*), Trm11 expression was analyzed by the same method.

Purification of tRNA^{Trp}. Total RNA was extracted from 2.0 g of cells by using Isogen II (Nippon Gene Co., Ltd.) in accordance with the manufacturer's protocol. The tRNA fraction was further purified by 10% PAGE (7 M urea). Transfer RNA^{Trp} was purified from the tRNA fraction by the solid-phase DNA probe method (21). The sequence of the DNA probe was complementary to G16 to A36 in tRNA^{Trp}: 5′-TGG AGC CCG CGA TGA TGG ACC AGG-biotin 3′.

Purification of human tRNA$^{Leu}{}_{CAA}$. Human cytoplasmic tRNA$^{Leu}{}_{CAA}$ containing m^5Cm was isolated from *ALKBH1* knockout cells as described previously (27).

Cyanoethylation of pseudouridines in tRNA. Five picomoles of isolated tRNA dissolved in 1 μl of Milli-Q water was mixed with 30 μl of 50% (vol/vol) ethanol–1.1 M trimethylammonium acetate (pH 8.6)–1 mM EDTA. After the addition of 4 μl of acrylonitrile (Wako Pure Chemical Industries), the solution was incubated at 70°C for 2 h. Cyanoethylated tRNA was collected by ethanol precipitation. The tRNA sample was treated with RNase T_1 digestion and then analyzed by liquid chromatography-mass spectrometry (LC/MS), as described below.

LC/MS. The isolated tRNAs were digested with RNase T_1 (Ambion) or RNase A (Ambion) and subjected to capillary LC–nano-electrospray ionization MS, as previously described (111, 112). For nucleoside analysis, 5 to 10 pmol of tRNA was digested by a 3-step reaction using nuclease P1 (Wako Pure Chemical Industries), phosphodiesterase I (Worthington Biochemical Corporation), and bacterial alkaline phosphatase (*Escherichia coli* C75) (TaKaRa Bio) (112). The digests were subjected to hydrophilic-interaction LC/MS analysis as described previously (113).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00448-19.

SUPPLEMENTAL FILE 1, PDF file, 3.6 MB.

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We declare no competing interests with respect to the work performed in the study.

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