Formation of m²G6 in Methanocaldococcus jannaschii tRNA catalyzed by the novel methyltransferase Trm14

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ABSTRACT

The modified nucleosides N²-methylguanosine and N²,N²-dimethylguanosine in transfer RNA occur at five positions in the D and anticodon arms, and at positions G6 and G7 in the acceptor stem. Trm1 and Trm11 enzymes are known to be responsible for several of the D/anticodon arm modifications, but methylases catalyzing post-transcriptional m²G synthesis in the acceptor stem are uncharacterized. Here, we report that the MJ0438 gene from Methanocaldococcus jannaschii encodes a novel S-adenosylmethionine-dependent methyltransferase, now identified as Trm14, which generates m²Ga at position 6 in tRNACys. The 381 amino acid Trm14 protein possesses a canonical RNA recognition THUMP domain at the amino terminus, followed by a c-class Rossmann fold amino-methyltransferase catalytic domain featuring the signature NPPY active site motif. Trm14 is associated with cluster of orthologous groups (COG) 0116, and most closely resembles the m²G10 tRNA methylase Trm11. Phylogenetic analysis reveals a canonical archaeal/bacterial evolutionary separation with 20–30% sequence identities between the two branches, but it is likely that the detailed functions of COG 0116 enzymes differ between the archaeal and bacterial domains. In the archaeal branch, the protein is found exclusively in thermophiles. More distantly related Trm14 homologs were also identified in eukaryotes known to possess the m²G6 tRNA modification.

INTRODUCTION

A large fraction of the over 100 modified nucleosides identified in RNA molecules are found in tRNA, substantially expanding the chemical diversity of the family (1,2). Methylation of the four canonical nucleotide bases at a wide variety of positions is particularly prominent among the known modifications (3). Among other functions, methylated tRNA bases are known to help stabilize tRNA structure (4), ensure decoding specificity during translation on the ribosome (5) and provide determinants for efficiency and selectivity in aminoacylation (6–9). All methyl groups added to tRNA bases are introduced by specific tRNA methyltransferase enzymes. In the great majority of cases, the methyl group is derived from S-adenosylmethionine (SAM), although examples are known in which folate and flavin adenine dinucleotide (FAD) are used instead (10). The SAM-dependent tRNA methyltransferases are divisible into five structural classes, each possessing a distinct α/β topology in the active site domain (11). Among these, the majority of the known enzymes possess the Class I structure featuring the ancient Rossmann fold.

Although the N²-methylguanosine modification has been found at positions 6, 7, 9, 10, 18, 26 and 27 of tRNA (1,12), the only modifying enzymes that have been characterized are those associated with methylation at positions G10, G26 and G27 (13–16). These three nucleotides are located in the tRNA vertical arm at the junction of the D and anticodon stems, where the structure deviates from canonical A-form helical geometry owing to a non-contiguous sugar–phosphate backbone and non-Watson–Crick hydrogen-bonding arrangement at the bridging 26–44 pair. The enzyme catalyzing dimethylation at G26, Trm1, produces the monomethylated

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species as a reaction intermediate, and possesses the Class I structure together with a unique 140 amino acid α/β C-terminal domain of unknown function (13,14). In at least one case, the *Aquifex aeolicus* Trm1, the enzyme exhibits multisite recognition properties: it is able to generate dimethylguanosine at both G26 and G27 (13). The archaeal enzyme catalyzing m2G10 formation, Trm11, possesses an N-terminal THUMP domain (found in thioeukine synthases, RNA methyltransferases and pseudouridine synthases) linked to a canonical Class I catalytic domain (15). Like m2G26 synthesis by Trm1, Trm11-catalyzed formation of m2G10 also proceeds through a monomethylated intermediate. In yeast, m2G10 formation requires a second protein, designated Trm112, and possibly additional factors as well (16). Interestingly, the sequence and structural features in the tRNA which are required for monomethylation of G10 and dimethylation of G26 are the same, suggesting independent evolution of Trm1 and Trm11 toward their distinct positional specificities.

We sought to further our understanding of m2G incorporation into tRNA by examining archaeal genomes for additional candidate enzymes. We focused our search primarily on methanogens, since biochemical studies have identified the presence of m2G in tRNA isolated from the hyperthermophiles *Methanocaldococcus jannaschii*, *Methanosococcus thermolithotrophicus*, *Methanococcus igneus* and *Methanopyrus kandleri* (17,18), the mesophiles *Methanococcus vanielli* and *Methanococcus maripaludis* (17), and the psychrotolerant *Methanococcus burtonii* (19). Among other Archaea, m2G has been detected in tRNA isolated from *Pyrococcus furiosus*, *Thermoproteus neutrophilus*, *Haloflexa volcanii* (previously named *Halobacterium volcanii*) and *Pyrolobus fumarii* (20–23). Study of archaeal tRNA modification has provided insight into the roles of the modified nucleosides in stabilizing tRNA structure at the elevated temperatures where hyperthermophiles flourish. In general, it appears that archaeal tRNA modifications are more similar to their counterparts in eukarya than in bacteria, yet are often simpler in structure than those present in either of the other domains (17,19,24,25).

Identification of novel tRNA methylases in methanogenic *M. jannaschii* of the MJ0438 gene product from *M. jannaschii* demonstrates that this protein catalyzes formation of m2G6 in *M. jannaschii* tRNA. This is the first identification of the enzyme responsible for m2G6 formation in tRNA.

**MATERIALS AND METHODS**

Expression and purification of recombinant Trm14

*Methanocaldococcus jannaschii* genomic DNA was purchased from the American Type Culture Collection, and used as template for polymerase chain reaction (PCR) amplification of the open reading frame corresponding to the MJ0438 gene, which we designate *trm14*. The primers used were: 5'-AAACTGCATAATGATTACTATGGTTA CACTATCC (forward primer) and 5'-CCGCTCGAGACGTAAATAAAAACACC (reverse primer). The reaction was carried out using 100 ng of genomic DNA, 20 μM of each primer (forward and reverse), 250 μM dNTPs, 10× Pfu turbo reaction buffer and 2.5 U of Pfu turbo DNA polymerase, at 51°C and 30s per cycle for a total of 32 cycles. The PCR product containing the *trm14* gene was digested with NdeI and XhoI, and inserted into the pet22b+ vector (Novagen) for expression of C-terminal His-tagged protein in *Escherichia coli* Rosetta2(DE3) pLysS cells. Cells were grown at 37°C in Luria Broth (LB) medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol.

Cells expressing Trm14 were recovered in a solution containing 50 mM NaH2PO4 (pH 7.8), 0.8 M NaCl, 15 mM β-mercaptoethanol and 10 mM imidazole, and then disrupted by sonication. The lysate was applied to a Ni-NTA agarose (Qiagen) column. Two wash steps were performed at increasing imidazole concentrations of 45 mM and 60 mM, prior to elution of Trm14 in buffer containing 250 mM imidazole. The enzyme was then dialyzed against a solution containing 50 mM NaH2PO4 (pH 7.8), 150 mM NaCl, 15 mM β-mercaptoethanol and 50% glycerol, and stored at −20°C. Trm14 was recovered.
at ~98% purity as judged on Coomassie-stained SDS–polyacrylamide gels, at a yield of roughly 2 mg of purified protein per liter of culture.

**Preparation of tRNA substrates**

Wild-type and mutant forms of *M. jannaschii* tRNA^Cys*, M. jannaschii* tRNA^Asp* and *M. jannaschii* tRNA^Pro*(TGG) were transcribed from synthetic duplex DNA templates. The templates were each first synthesized from two overlapping synthetic deoxyribonucleotides (purchased from Fisher Operon). For the wild-type tRNA^Cys* gene, the oligonucleotides used were: 5'-AAAT TCC TGCA GTCT GCCT ATGC TAT GGC CGG GGT AGTT CA TGAG GCC GAG CAG CGG ACT G (forward primer), and 5'-TGG AGC CGG GGG GATTT GAC TAT AGC CGG GGT AGTT CTA GCC GGT ACT AGG CAG CGG ACT G (reverse primer), where the underlined portions represent the overlap region. Primers used for the other tRNAs are provided in Supplementary Data. Overlapping DNAs were extended using Klenow fragment of *E. coli* DNA polymerase I, as described (33), and were recovered by ethanol precipitation. In vitro transcription reactions were then performed as described (33). Typically, 0.1 mg of DNA template was used in the transcription reaction to generate 1 mg of tRNA. tRNA^Cys* was purified by gel extraction and stored as an ethanol precipitate or in purified water.

**In vitro methylation reactions**

Except where otherwise specified, methylation reactions included the purified Trm14 protein at a final concentration of 10 μM. Reactions were performed at 52°C in 0.2 M Tris–HCl (pH 8.0), 0.8 mM DTT, 1.2 mM MgCl₂, 20 mM KCl, 4.8 μg/ml bovine serum albumen (BSA), using 1 μM *M. jannaschii* tRNA^Cys* transcript as the substrate and 100 μM SAM [a mixture containing 7.55 μM of 68 Ci/mmol [3H]SAM (Perkin Elmer) and 92.45 μM unlabelled SAM (Sigma)]. Prior to methylation, the tRNA was heated at 85°C for 3 min and allowed to renature by addition of 5 mM MgCl₂ followed by slow cooling to room temperature. At various time points, 5 μl aliquots were removed, precipitated with 5% (w/v) trichloroacetic acid (TCA) on filter pads, washed with ethanol and ether and air-dried before scintillation counting. These methylation conditions were also used to assay methylation of tRNA^Cys* mutants and WT tRNA^Pro* and tRNA^Asp* substrates.

**Modified nucleotide analysis**

Radiolabeled tRNA^Cys* was synthesized in vitro as described above, except that the transcription reactions also included 125 μCi [α-32P] GTP (3000 Ci/mmol). Trm14 reactions were then performed, and the labeled, methylated tRNA recovered by phenol/chloroform and ethanol precipitation. Thirty μg of methylated tRNA was resuspended in 5 μl water and digested with 3 μl nuclease P1 (1 mg/ml) at 37°C for 1 h to generate 5’-phosphorylated nucleotides. A 2 μl aliquot was then applied to a thin layer cellulose (TLC) plate (Sigma, 801 063). Chromatography in the first dimension was carried out in isobutyric acid/ammonia/water (66/1/33 [v/v/v]) (solvent A), while the second dimension was developed with isopropanol/concentrated HCl/water (68/18/14 [v/v/v]) (solvent C). TLC plates were analyzed by phosphorimaging, and the modified and unmodified nucleotides were identified using reference maps (34).

For liquid chromatography–mass spectrometry (LC–MS) analysis of modified nucleosides, nuclease P1 (Sigma), snake venom phosphodiesterase I (Worthington Biochemical Corporation) and Antarctic phosphatase (New England Biolabs) were used to digest Trm14- reacted transcripts and control unmethylated transcripts to nucleosides. The nucleosides were separated using a Hitachi D-7000 HPLC with a Hitachi L-7400 UV detector at 0.3 ml/min at room temperature on an LC-18S 2.1 × 250 mm column from Supelec using a gradient of 5 mM ammonium acetate pH 5.3 and acetonitrile:water (40:60 v/v) as described (35). The eluent from the column was split with two-thirds flowing into the UV detector and one-third flowing into a Thermo LTQ-XL (Waltham, MA, USA) equipped with an electrospray ionization (ESI) source. Mass spectra were recorded in the positive ion mode at a capillary temperature of 275°C, spray voltage of 3.7–4 kV and sheath gas, auxiliary gas, and sweep gas set to 45, 25 and 10 arbitrary units, respectively.

**LC–MS/MS analysis of RNase digests**

Transcripts were incubated with 0.01 U of RNase A per microgram of RNA in 20 mM ammonium acetate for 2 h at 37°C. Digestion products from 1 μg of RNA were separated using a Thermo Surveyor HPLC system with an Xterra C18 1.0 × 150 mm column (Waters) at room temperature with a flow rate of 40 μl/min. Before each run the column was equilibrated for 15 min at 95% Buffer A [200 mM 1,1,1,3,3,3-hexafluoropropanol (HFIP), 8.15 mM triethylamine (TEA)] and 5% Buffer B (200 mM HFIP, 8.15 mM TEA:methanol 50:50 v/v). Both buffers were adjusted to pH 7.0. The gradient used was 5% B for 5 min, 30% B at 7 min and 95% B at 50 min and held at 95% B for 5 min. The eluent was directed into a Thermo LTQ-XL. Operating parameters were capillary temperature of 275°C, spray voltage of 4.5 kV, sheath gas at 25 arbitrary units, auxiliary gas at 14 arbitrary units and sweep gas to 10 arbitrary units. Collision-induced dissociation (CID) tandem mass spectrometry (normalized collision energy 35%) was used in data-dependent mode to obtain sequence information from the RNase A digestion products. The data-dependent scan was recorded based on the most abundant ion, and each ion selected for CID was analyzed for 30 scans or 30 s before it was added to a dynamic exclusion list for 30 s.

**Cell strain and growth**

*Methanocaldococcus jannaschii* strain JAL-1 was obtained from the Oregon Collection of Methanogens (OCM 168 = DSM 2661) and maintained in the laboratory as frozen stocks in DSM 282 medium with 25% glycerol. Cultures were routinely grown in liquid medium (DSM 282) prepared anaerobically by a modification of the Hungate technique (36). Medium was dispensed in 25 ml aliquots into 160-ml serum bottles, and sealed under an

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N₂–CO₂ (4:1) atmosphere with butyl rubber stoppers secured with aluminum crimp seals. Na₂S·9H₂O was added to a final concentration of 0.025% immediately prior to inoculation. After inoculation the gas phase was replaced with H₂–CO₂ (80:20) pressurized to 101 kPa. Cultures were incubated on a rotary shaker at 250 rpm at 83°C. Growth was routinely monitored by measuring methane production and gas phase was repressurized with H₂–CO₂ (80:20) whenever a vacuum was detected from depletion of substrate. For preparation of cell material, cultures were scaled up by inoculating six 25 ml cultures into 161 of selenium enriched medium in a 201 Bioflo IV bioreactor (New Brunswick Scientific, Edison NJ) (37). The culture was subsequently transferred anaerobically to 2101 of medium in a 2501 bioreactor (New Brunswick Scientific). Both bioreactors were maintained at 83°C and pH 6.0 with a H₂–CO₂ (80:20) flow-rate of 0.2 vvm at 1.7 × 10⁵ Pa. Cells from a 2501 fermentor were harvested in late exponential growth by centrifugation in a 21 capacity continuous flow centrifuge (CEPA Carl Padberg Zentrifugenbau GmbH, Germany) and stored in liquid nitrogen.

Methylation activity in cell extracts

*Methanocaldococcus jannaschii* cells (2.5 g) were resuspended in 3 ml of a solution containing 20 mM Tris–HCl (pH 7.4) and 50 mM NaCl, and were frozen overnight at −20°C. The cells were thawed, resuspended and sonicated at 50% duty cycle for 2 min. The lysate was then ultracentrifuged at 80,000 rcf using a 70.1 Ti rotor (Beckman Coulter). The supernatant was recovered and concentrated to 2 ml using spin columns with a 30 000 Da cut-off to partially remove endogenous tRNA, thereby reducing background methylation activity. The extract was then dialyzed into a buffer containing 10 mM Tris–HCl (pH 7.3), 20 mM β-mercaptoethanol, 1 mM EDTA, 4 mM MgCl₂ and 15% glycerol for 8 h at 4°C, and concentrated to 100 mg/ml protein, as estimated from absorbance at 280 nm.

Methylation reactions were conducted in 50 µl volumes using conditions detailed above, 1 µM WT or G6A/C67U tRNA<sup>Cys</sup> transcript as substrate and 200 µg (2 µl) of protein from *M. jannaschii* extract as the source of Trm14 activity. *Escherichia coli* S100 cell extracts were prepared by standard approaches, and methylation reactions were conducted using extract equivalent to 500 µg protein, 40 U of anti-RNAsin, 3 µM WT or G6A/C67U tRNA<sup>Cys</sup> and 50 µM SAM in 50 µl reactions carried out at 37°C (38).

Bioinformatics

Initial comparative analysis of a limited subset of methanogen genomes was performed using STRING (v 6.2) (39). Sequence homology searches to the omniome or selected subsets of the omniome were performed with BLAST (40). For phylogenetic analysis, the multiple sequence alignment for a subset of proteins within COG 0116 was carried out using ClustalW (41). The phylogenetic tree based on these sequences was constructed with MEGA5 (42), using the neighbor-joining method with the BioNJ(JTT) model. The bootstrap test (1000 replicates) was performed to validate the stability of the nodes.

RESULTS AND DISCUSSION

Identification, recombinant expression and purification of Trm14

A bioinformatics-based approach to identify new putative methylase activities in methanogens was taken. To link the search to unique aspects of protein synthesis in these organisms, we elected to probe genomes for the presence of RNA methylases that covery with the presence or absence of a canonical CysRS enzyme. The initial search in six methanogen genomes was carried out using STRING, a bioinformatics resource developed to identify physical and functional interactions among proteins (39). The genomes probed were: *M. jannaschii*, *M. kandleri*, *M. thermotrophicus*, *M. mazei*, *M. acetivorans* and *M. maripaludis*. SQL (structured query language) queries within STRING were used to interrogate these six genomes to determine which COGs were present in all three of the organisms *M. jannaschii*, *M. kandleri* and *M. thermotrophicus*, and absent in each of the organisms *M. mazei*, *M. acetivorans* and *M. maripaludis*. The latter group possesses both SepRS and CysRS, while the former group possesses only SepRS.

Eight COGs were identified by this approach, providing a basis for abundant further analysis to identify aspects of methanogen metabolism related to how cysteine is incorporated into proteins. For this study, we focused on COG 1818, annotated as a predicted RNA binding protein containing the THUMP domain. Seven proteins in *M. jannaschii*, *M. kandleri* and *M. thermotrophicus* are found in COG 1818. All seven proteins were then used as queries in BLAST searches of all methanogen genomes. The search in which a COG 1818 protein from *M. kandleri* (2320.Q8TVD0) was used as query revealed a predicted N⁶-adenine specific RNA methylase containing a THUMP domain (MK0969) also from *M. kandleri*. Subsequent BLAST searches of all methanogen genomes using MK0969 as query sequence then revealed that a highly similar protein is present in a subset of these organisms, all of which lack CysRS (Supplementary Data). Some methanogens, such as *M. burtonii* and *M. thermotrophicus*, lack both CysRS and the MK0969 homolog. An MK0969 homolog is never present when both SepRS and CysRS are found in a methanogen genome. These characteristics suggested that the function of the protein might be related to the loss of CysRS from a methanogen genome. Phylogenetic analyses suggest that the last universal common ancestor (LUCA) possessed both CysRS and SepRS; it appears that SepRS has since been lost everywhere except in most methanogens and the related *A. fulgidus* (43).

In this manner, we identified eight methanogens possessing a predicted RNA methylase homologous to MK0969, all of which are thermophiles or hyperthermophiles (Supplementary Data). These proteins are members of COG 0116. We elected to study the MJ0438 homolog from *M. jannaschii*, because this is the best-studied
methyltransferase activity of Trm14

We tested the activity of recombinant, purified Trm14 on a variety of DNA and RNA substrates, using an assay in which the methyl group from [3H]SAM is incorporated into nucleic acid, and the acid-precipitable labeled product then isolated on filter paper and quantitated by liquid scintillation counting. As expected given the presence of a THUMP domain in the protein, no activity was detected toward M. mazei tRNA even at very high concentrations of both Trm14 and substrate (10 μM Trm14; 1 μM tRNA) (Figure 1B).

In contrast, transfer of [3H]-methyl groups from SAM was readily detected using a purified and renatured M. jannaschii tRNA\textsuperscript{Cys} transcript as substrate (Figures 1B, 1C and 2). Methylation reaction conditions were optimized with respect to the identity and concentration of the buffer, pH of the reaction and temperature: low methylation activity was observed at 37°C, while the activity was considerably higher at 52°C, the highest temperature compatible with maintenance of tertiary structure in the tRNA transcript. Under conditions of enzyme molar excess, we observed that the activity toward M. jannaschii tRNA\textsuperscript{Cys} approaches the incorporation of one methyl group per tRNA molecule (Figures 1B and 2). An M. jannaschii tRNA\textsubscript{Asp} transcript also could be methylated, albeit with reduced efficiency as compared with tRNA\textsuperscript{Cys} (Figure 2C). In contrast, methylation of tRNA\textsuperscript{Pro(TGG)} was detectable only slightly above background levels. Together, the data show that Trm14 is able to specifically catalyze methyl group incorporation into tRNA in vitro, with variation in the efficiency of methylation depending on the identity of the isoacceptor species.

To more rigorously assess the function of Trm14 as a tRNA methylase, we carried out methylation reactions using M. jannaschii tRNA\textsuperscript{Cys} at a variety of protein concentrations and with saturating concentrations (50 μM) of SAM (Figure 2A and B). Only very weak activity could be detected under multiple-turnover conditions corresponding to 100 nM enzyme and 1 μM tRNA. However, robust methylation was observed when the enzyme is present in molar excess over its tRNA substrate. The concentration of Trm14 was varied while maintaining tRNA\textsuperscript{Cys} at 1 μM, and reaction time courses were monitored in each case (Figure 2A). Replot of the variation in the extracted \(k_{\text{obs}}\) with Trm14 concentration shows saturation, with a maximum value for the rate constant of about 0.05 min\(^{-1}\) (Figure 2B).

The robust concentration-dependent behavior of recombinant Trm14 in catalyzing methylation of tRNA\textsuperscript{Cys} provides substantial confidence that this activity is physiologically relevant. The data also suggest that Trm14 may function as a single-turnover catalyst in vivo. This is consistent with the notion that tRNA structure in hyperthermophiles may be stabilized by protein binding: the relatively high intracellular Trm14 concentrations that may be needed for tRNA methylation may also serve to protect the molecule from thermal denaturation (25). However, an alternative rationale for the very weak

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**Figure 1.** (A) SDS gel showing purified, recombinant Trm14 produced from E. coli. (B) Time courses for methylation of nucleic acid substrates by Trm14. The DNA substrate consisted of a 56-mer oligodeoxyadenosinate. tRNA was purified from M. mazei cells. (C) Cloverleaf secondary structure representation of M. jannaschii tRNA\textsuperscript{Cys}.

methyltransferase activity was detected toward a 56 nt single-stranded oligodeoxynucleotide (Figure 1B). Plasmid DNA did not function as a Trm14 substrate, nor did a 37-mer chemically synthesized RNA corresponding to a bacterial tRNA\textsuperscript{Gln} 3'-half molecule (data not shown). To examine whether Trm14 possesses activity toward rRNA, total native RNA was isolated from M. mazei, which lacks a Trm14 homolog, and the rRNA was separated from tRNA by gel filtration chromatography. Large and small-subunit RNAs in M. mazei and M. jannaschii are 77% and 87% identical, respectively. No activity was detected toward M. mazei rRNA even at very high concentrations of both Trm14 and substrate (10 μM Trm14; 1 μM tRNA) (Figure 1B).

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activity of Trm14 under steady-state conditions is that another factor or condition present in vivo might be missing in these in vitro experiments. For example, the archaeal protein from the hyperthermophile Pyrococcus abyssi increases the substrate specificity of a tRNA m^5C methyltransferase (45); a homolog of this protein exists in the M. jannaschii genome. Comparable time courses for SAM-dependent methyl group incorporation were reported for m^2G26 and m^2G10 formation by Pyrococcus horikoshii Trm1 and P. abyssi Trm11, respectively (14,46).

Trm14 is a novel m^2G6 tRNA methylase

We employed a combination of LC–MS/MS analysis and two-dimensional thin-layer chromatography (2D-TLC) to identify the product of methyl transfer by Trm14. Methylated and unmethylated tRNA^Cys transcripts were first digested to nucleosides with RNase P1, snake venom phosphodiesterase and Antarctic phosphatase, and the resulting mixture was separated and analyzed by LC–MS. Splitting the flow from the high pressure liquid chromatograph (HPLC) into a UV monitor and mass spectrometer allowed monitoring of elution time together with the mass spectra of the peaks (35). Nine UV-absorbing peaks were identified from the methylated tRNA (Figure 3A): the four standard nucleosides of RNA and DNA, together with one modified nucleoside. The modified nucleoside eluted at 26.07 min with a molecular mass of 297 Da ([M+H]^+ m/z 298). No other species at this m/z were detected in the chromatogram (Figure 3B). The selected ion chromatogram (SIC) of the modified base ion, m/z 166 (BH_3^+) was also recorded (Figure 3C). The mass difference of 132 u between the molecular ion and base ion is consistent with location of the modification on the base and not the ribose of the nucleoside. These m/z values and the elution time best match a methylated guanosine with an expected m/z of 298 for the nucleoside and 166 for the base. Either m^1G or m^2G match the elution time within the experimental precision of the measurements.

To distinguish whether the modified base is m^1G or m^2G, we employed 2D-TLC analysis. The M. jannaschii tRNA^Cys transcript was synthesized by T7 RNA polymerase in reaction mixtures supplemented with [α-^32P] GTP for internal labeling. The transcript was purified, refolded in vitro and methylated by Trm14. Next, the methylated transcript was digested with P1 nuclease to generate 5’-monophosphorylated nucleosides, which were then separated using two-dimensional thin-layer chromatography (2D-TLC) (Figure 4). Comparison of the migration patterns of labeled 5’-monophosphorylated nucleosides in unmethylated and methylated samples revealed the presence of a single modified base, and comparison with established reference maps in this solvent system demonstrates that the modified base is m^2G (34). All of the methylated guanosine nucleosides found in unfractionated M. jannaschii tRNA hydrosylates, and that can be generated by the activity of a single methyltransferase, are present on the reference maps (17). The plateau methylation level equivalent to transfer of one methyl group per tRNA^Cys substrate molecule (Figure 2) is consistent with identification of a monomethylated product.

To identify the position of m^2G formation in the tRNA, transcripts were digested with RNase A and subsequently analyzed by LC–MS/MS (47–52). Upon elution from the C18 column, the digestion products were visualized via the total ion chromatogram (TIC) (Figure 5A). Comparing the TIC of the unmodified and modified RNAs (Figure 5A and 5B) revealed an additional digestion product in the modified sample eluting between

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Figure 2. (A) Kinetic analysis of M. jannaschii Trm14 toward an unmodified tRNA^Cys transcript. Single-turnover reaction time courses carried out at 50 μM SAM, 1 μM tRNA^Cys transcript and varying concentrations of Trm14. For each time course k_0 is derived by fitting the data to a single exponential function. (B) Replot of k_0, from part A, as a function of enzyme concentration. (C) Comparative methylation of tRNA transcripts by Trm14.
22 and 23 min that is not present in the unmethylated control. The major peak eluting around 22 min in both sample was analyzed (Figure 5C) and found to contain two digestion products that co-elute with m/z 851.25 and 654.75 and correspond to the doubly charged ions for GGGGUp and AAAUp, respectively (Figure 5D).

The additional digestion product eluting between 22 and 23 min that appears when the RNA is treated with Trm14 was analyzed (Figure 5E) and was found to contain a doubly charged ion at m/z 858.17 (Figure 5F). The difference in m/z values between the closely eluting m/z 851 peak (GGGGUp) and this m/z 858 peak is...
consistent with the addition of a methyl group to the GGG GUp digestion product. To determine the location of this methyl group, data-dependent CID for tandem mass spectrometry (LC–MS/MS) was performed. Fragmentation of the m/z 851 digestion product was consistent with the sequence GGGGUp with the identification of many of the expected fragmentation products (y-, w- and c-ions) (Figure 6A). Fragmentation of the m/z 858 digestion product shows a mass shift in the c3, c4, y3, w3 and w4 fragments that is consistent with the addition of 14 u on the guanosine in the third position in the RNase A digestion product (Figure 6B). Combined with the previous nucleoside analyses, these results are consistent with the sequence GGm2GGUp for this RNase digestion product. The digestion product with the sequence of GGm2GGUp can only be located in one part of the sequence in this tRNA transcript, position 6 of the tRNA (Figure 6C).

To further confirm that Trm14 catalyzes m2G formation at position 6 of tRNA^Cys, we synthesized a variant M. jannaschii tRNA^Cys transcript incorporating A6-U67 in place of the wild-type G6-C67 base pair. Methylation time courses with 1 mM mutant tRNA, saturating levels of SAM and molar excess of Trm14, conditions identical to those used for the wild-type transcript, showed extremely low activity (Figure 7). In contrast, the A6-U67 tRNA variant was an excellent substrate for M. jannaschii Trm5, which requires a properly folded tertiary core region for efficient formation of m1G37 (data not shown) (38). Thus, as expected, replacement of the G6-C67 acceptor stem base pair with the isosteric A6-U67 does not compromise the integrity of the tRNA structure. These experiments support the identification of m2G6 as the site of Trm14 methylation. Further, Trm14-catalyzed methylation of tRNA^Asp is consistent with the presence of G6 in this species, while the extremely weak activity toward tRNA^Pro likely arises from the presence of U6 in this isoacceptor (Figure 2C).

A tRNA^Cys m2G6-forming activity in native M. jannaschii cell-free extract

To further validate the assignment of MJ0438 as a tRNA methylase, we examined whether cell-free lysates of M. jannaschii possess an equivalent activity. We cultivated M. jannaschii strain JAL-1 under anaerobic conditions in a 250 l bioreactor, recovered cells by centrifugation, and prepared S100-fractionated lysates by sonication. Background incorporation levels are very low using this extract as the source of enzyme, 3H-labeled SAM as methyl-group donor, and no added tRNA (Figure 8A). Inclusion of 1uM M. jannaschii tRNA^Cys transcript in the reactions then revealed that the extract possesses...

Figure 5. LC–MS analysis of RNaseA digestion products of Trm14-reacted and control unreacted tRNA^Cys transcripts. (A) TIC of RNase A-digested RNA transcripts. (B) TIC of RNase A-digested RNA transcripts treated with Trm14. There is clearly an additional digestion product (asterisks). (C) SIC of the unmodified digestion product with the sequence GGGGUp at m/z 851 (doubly charged). This product was found in the Trm14 treated and untreated samples. (D) Mass spectra of the digestion products at the elution time of the m/z 851 product. There is a digestion product with the sequence AAAUp that co-elutes with m/z 654.75 (doubly charged). (E) SIC of the modified digestion product with the sequence GGm2GGUp at m/z 858 (doubly charged). (F) Mass spectra of the digestion products at the elution time of the m/z 858 product.
robust tRNA methylase activity. Importantly, plateau methylation levels were decreased nearly 3-fold when the A6-U67 tRNA Cys variant was substituted for WT tRNACys as the substrate. Thus, a substantial portion of the activity in native cell lysates is sensitive to the identity of the base pair at position 6–67 in a manner consistent with the \textit{in vitro} activity of Trm14. The remaining SAM-dependent activity toward the A6-U67 transcript could be associated with formation of m1A, m5C, m1G, m6A, m2-2Go rm 2G, as all of these modified bases were previously identified in \textit{M. jannaschii} tRNA (17). These findings suggest that Trm14 is expressed \textit{in vivo} and is responsible for a significant portion of the observed activity.

As a further control, we tested an \textit{E. coli} S100 extract for its capacity to methylate an exogenously added \textit{M. jannaschii} tRNACys transcript. While this preparation is indeed capable of heterologous methylation at levels \textasciitilde2-fold lower than observed for \textit{E. coli} tRNACys, the level of methyl group incorporation is insensitive to the identity of the 6–67 bp (Figure 8B). This is consistent with the known absence of methylation at the 6–67 bp in any \textit{E. coli} tRNA (1), and with our inability to detect the presence of a Trm14 homolog in the genome.

**Phylogenetic distribution of Trm14-like proteins**

All proteins identified as members of COG 0116 (31) encompass two domains: a THUMP (thiouridine synthases, RNA methyltransferases and pseudouridine synthases) domain (32), and a C-terminal SAM-dependent methyltransferase Rossmann fold. A conserved polypeptide 40 amino acids in length is present at the N-terminus, but this portion of the enzyme possesses no detectable sequence similarity with other proteins. Alignment of a subset of COG 0116 proteins, together with other candidates identified in BLAST searches of the omniome using \textit{M. jannaschii} Trm14 as the query sequence, reveals similarities across the entire structure (Figure 9).
A phylogenetic tree of the COG 0116 family based on this alignment clearly distinguishes three main branches corresponding to the bacterial, archaeal and eukaryotic domains (Figure 10). The eukaryotic branch is more distantly related to the bacterial or archaeal branches than the latter two branches are to each other.

The separation between the bacterial and archaeal branches, and the closer connection of the eukaryotic branch to the archaeal, suggests a canonical vertical gene flow pattern. However, there are several exceptions. First, in the hyperthermophilic bacterium Thermus thermophilus, the protein that is most similar to *M. jannaschii* Trm14 is more closely related to proteins in the archaeal branch than to those of other bacteria (29% identity to the *M. jannaschii* ortholog; Figures 9 and 10). Second, the tightly grouped eukaryotic proteins depicted in Figure 10 are confined to chordates. However, BLAST searches with *M. jannaschii* Trm14 as query also detect other eukaryotic sequences, including from the unicellular species Paulinella chromatophor and Thalassiosira pseudonana, the nematode Caenorhabditis remanei, and the plants Ricinus communis and Populus trichocarpa. The sequences of these proteins are not closely related to those of the chordates, and are instead dispersed in the bacterial domain. If all these proteins are indeed homologs with a common evolutionary origin, then significant horizontal gene transfer may have occurred. Further analysis will be required to examine this possibility in greater depth.

The Trm14 THUMP domain has previously been found in enzymes modifying tRNA at diverse sites. At position 8, both an archaeal cytosine deaminase activity and ThI-catalyzed 4-thiouridine formation are associated with this domain (53,54). The tRNA positional specificity of THUMP domains also includes position 12 (N4-acetylcytidine formation by Tan1) (55), position 55 (pseudouridine formation by human Pus10) (56) and position 10 (m2G10 formation by Trm11) (15). Interestingly, m2G26 formation is catalyzed by a Rossmann-fold class methyltransferase, Trm1, which does not possess a THUMP domain (13,14,46). In general, THUMP domain enzymes have previously been found associated only with modification in the globular tRNA core, but target diverse positions within this region. The association of THUMP with catalysis of m2G6 formation by Trm14, then, expands its known positional specificity. A separately expressed THUMP domain possessed only weak tRNA binding activity (57); in general, how THUMP functions to target a wide variety of modification activities throughout the globular core region remains unknown.

Within the Trm14 catalytic domain, the alignments reveal conservation of the SAM binding site, which includes residues in motifs I, II and III, as well as the conserved catalytic sequence NPPY in motif IV. The common motifs...
of the amino methyltransferase family are present in the sequential order X, I, II, III, IV, V, VI, VII and VIII, indicating that the COG 0116 proteins belong to the \( g \) class of these enzymes (58). More detailed examination of the alignment shows that the proteins in the archaeal and bacterial branches possess some consistent distinctions in the conserved motif sequences. In motif II, while proteins from both branches possess a conserved negatively charged residue (usually Asp) that interacts with the SAM ribose group, most members of the bacterial branch contain Asp-X-Asp/Glu while the archaeal enzymes lack the second negatively charged residue. In motif IV, almost all Trm14 proteins contain the signature NPPY motif of the group \( g \) aminomethyltransferases, which is followed by Gly. The exceptions include \( T. \) thermophilus and the \( Pyrococci \) species with ‘NPPH’ and ‘NLPY’ as the catalytic motifs, respectively. Finally, motif V contains the consensus Leu-Tyr-X-X-Phe/Leu/Ile/Met. This motif can be detected in most members of the archaeal branch, but the slightly different signature Leu-Tyr-X-X-(Phe/Leu/Ile/Met)-Gly is present in the bacterial branch, with the Gly particularly well conserved. The Phe in motif V, which directly contacts the adenine moiety of SAM, is sometimes present when a spatially conserved Phe is missing at the position immediately N-terminal to the well-conserved Cys–Gly–Ser–Gly motif I sequence. Such replacement, when it occurs in the Trm14 family, is limited to the bacterial enzymes. The other common catalytic motifs III, VI, VII, VIII and X are not clearly distinguished between the two branches.

Among known tRNA methylases, the closest match of MJ0438 (18–26% sequence identity) is to the \( m^2_g \)A\(^{10} \)-forming Trm11 tRNA methylase, for which the ortholog exists in \( M. \) jannaschii (MJ0710) (40). MJ0710 and MJ0438 are similarly organized with N-terminal THUMP and C-terminal catalytic domains, but differ in the motif IV active-site sequence motif (DPPY in Trm11; NPPY in MJ0438). The phylogenetic distribution of Trm14 differs from that of Trm11, since the latter is found in Archaea and Eukarya only, and is absent in Bacteria. No crystal structure of Trm11 is yet available.

Figure 9. Multiple sequence alignment of Trm14-like proteins. The positions of the THUMP domain and the motifs in the class I methyltransferase catalytic domain (X, I, II, III, IV, V, VI, VIII) are indicated. Accession numbers for sequences are provided in the Supplementary Data.
Functional roles of Trm14-like proteins

*M. jannaschii* Trm14 is tightly grouped with likely orthologs in the *Methanocaldococcus* family (65–87% sequence identities), and with other Archaea such as *A. fulgidus*, *Acropyrum pernix*, *M. kandleri*, *P. abyssi*, *P. horikoshii* and *Nanoarchaeum equitans* (30–49% identities). Further, all organisms in the archaeal clade that possess Trm14 are thermophiles. Based on the high degree of sequence similarity, we expect that the Trm14 function in catalyzing tRNA m^2^G6 formation is very likely conserved among these archaeal proteins, and may play a role in stabilizing the structure of the RNA. The position of the modification appears unusual in this respect, since previously well-characterized examples of modification-derived thermal protection in tRNA are for nucleotides in the T-loop/D-loop interaction region at the outside globular corner of the L-shape (25). Possibly, m^2^G6 may function synergistically with these core-region modifications to stabilize the molecule against thermal stress.

Another possibility is that Trm14-catalyzed m^2^G6 formation in tRNA^Cys^ may play a role in modulating the aminoadenylation efficiency of SepRS. It is already known that the presence of m^3^G37 in the tRNA^Cys^ anticodon loop enhances aminoadenylation by *M. jannaschii* SepRS, but m^2^G6 is present in tRNA^Cys^ for bacteria known to lack m^2^G6 in tRNA, the Trm14-like ortholog of the thermophilic archaeal enzymes. However, evidence that function may diverge in the bacterial domain is available from tRNA sequence information. While sequences determined at the RNA level are available for a variety of bacteria (1), the only example known in which m^2^G6 is present in tRNA is *T. thermophilus*, which possesses a Trm14-like protein that groups with the archaeal clade (Figure 10). This concordance suggests that the *T. thermophilus* protein is an ortholog of the thermophilic archaeal enzymes. However, for bacteria known to lack m^2^G6 in tRNA, the Trm14-like protein that we have identified must possess a different function. An interesting example is provided by *E. coli* and some related bacteria. In these organisms, the COG0116 protein is much larger than *M. jannaschii* Trm14 and its related orthologs (Figure 9). The *E. coli* protein, the product of the *ycbY* gene, possesses an N-terminal THUMP domain followed by two consecutive catalytic
domains belonging to COG 0116 and, in the C-terminal portion of the protein, to COG 1092 (59). Knockout of *ycbY* (now renamed *rlmL*) showed that the enzyme catalyzes m^2^G2445 formation in 23S rRNA *in vivo*. The COG 1092 family contains several representatives in *M. jannaschii* (MJ1653 and MJ1649, each unlinked to MJ0438), and there are also several examples in which it is known that this protein methylates rRNA (60). Hence, the COG 0116 portion of *E. coli rlmL* may possess an entirely different function or may assist the rRNA methylation in an unknown way. No other members of the COG 0116 family from the archaeal, bacterial or eukaryal domains have been characterized.

The m^2^G6 modification has also been found in the tRNA of eukaryotes (1), including chordates such as *Homo sapiens* and *Xenopus laevis* that possess Trm14-like proteins more distantly related to the archaeal and bacterial enzymes. The chordate enzymes possess an additional 20–40 amino acid extension at their N-termini, and also conserve several other structural features in the catalytic domain not present in the bacterial and archaeal proteins (Figure 9). Further, these proteins either possess variants of the NPPY motif with substitutions at one or two positions, or do not conserve any of the four amino acids. The precise role of the active-site motif D/N/S-PP-Y/W/F/H is unknown, however, and the lack of conservation of any titratable side-chain group suggests that it does not directly participate in acid-base catalysis (3,61,62). Thus, the precise sequence of this motif has no clear predictive value, certainly not with respect to RNA substrate specificity. While the presence of m^2^G6 in chordate tRNA is provocative, direct experimental characterization of the Trm14-like protein in one or more of these organisms is required to assess whether it is indeed responsible for the activity. Previous studies monitoring formation of m^2^G6 in *Xenopus* tRNA *in vivo* showed that the activity is insensitive to alterations in the tRNA tertiary structure (63). In general, the identification of archaeal and candidate eukaryal m^2^G6-forming enzymes opens the possibility for more detailed structure–function studies to address questions of catalytic mechanism and tRNA sequence requirements for function. The possible roles of m^2^G6 in serving as a modulator of elongation factor affinity for aminoaacyl-tRNA or to regulate function on the ribosome are also entirely unknown at this time.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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