Identification and Characterization of the *Thermus thermophilus* 5-Methylcytidine (m\(^5\)C) Methyltransferase Modifying 23 S Ribosomal RNA (rRNA) Base C1942*§

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Line H. G. Larsen‡, Anette Rasmussen‡, Anders M. B. Giessing‡, Gerwald Jogi§1, and Finn Kirpekar‡2

From the ‡Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark and the §Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912

Background: The enzymes methylating C1942 and C1962 in 23 S ribosomal RNA of *Thermus thermophilus* remained to be identified.

Results: The methyltransferase targeting C1942 was identified and characterized with respect to substrate, exact product, and structure.

Conclusion: The methyltransferase has structural similarities with previously characterized methyltransferases producing 5-methylcytidine and 5-methyluridine.

Significance: The structure of RNA-methylating enzymes is important in the delineation of their evolution.

Methylation of cytidines at carbon-5 is a common posttranscriptional RNA modification encountered across all domains of life. Here, we characterize the modifications of C1942 and C1962 in *Thermus thermophilus* 23 S rRNA as 5-methylcytidines (m\(^5\)C) and identify the two associated methyltransferases. The methyltransferase modifying C1942, named RlmO, has not been characterized previously. RlmO modifies naked 23 S rRNA, but not the assembled 50 S subunit or 70 S ribosomes. The x-ray crystal structure of this enzyme in complex with the S-adenosyl-L-methionine cofactor at 1.7 Å resolution confirms that RlmO is structurally related to other m\(^5\)C rRNA methyltransferases. Key residues in the active site are located similar to the further distant 5-methyluridine methyltransferase RlmD, suggestive of a similar enzymatic mechanism. RlmO homologues are primarily found in mesophilic bacteria related to *T. thermophilus*. In accordance, we find that growth of the *T. thermophilus* strain with an inactivated C1942 methyltransferase gene is not compromised at non-optimal temperatures.

All living organisms and even organelles contain posttranscriptional modifications in their ribosomal RNA (rRNA). Posttranscriptional modifications have been mapped completely in yeast mitochondria (1) and in the *Escherichia coli* large ribosomal subunit rRNA (2, 3), and extensive maps exist for human, *Xenopus*, and budding yeast large ribosomal subunit rRNAs (4–7).

Posttranscriptional modifications are introduced by target-specific enzymes in eubacteria, whereas the most common modifications in eukaryotes, pseudouridinylation and 2′-O-ribose methylation, rely on shared enzyme systems that are guided to the target by sequence-specific small nucleolar RNAs (8–11). RNA-guided pseudouridinylation and 2′-O-ribose methylation of rRNA are also found in archaea (12, 13). Despite the different modification systems, there is a significant degree of phylogenetic conservation of modification site and modification nature in large ribosomal subunit rRNAs. This is exemplified by three pseudouridines (Ψ) in helix 69 (*E. coli* secondary structure nomenclature and nucleotide numbering used as default in this work) and ribose methylations of G2251 and U2552, which are found across all domains of life (6, 14–16).

The function of large ribosomal subunit RNA modifications appears quite diverse. The above mentioned Ψs in helix 69 are only of marginal importance in *E. coli* and *Salmonella* (17), whereas these Ψs affect protein synthesis fidelity in yeast (18). Other 23 S rRNA modifications have effects on antibiotic resistance (19), polypeptide elongation rate (20), ribosome assembly (21), and translational arrest (22) in *E. coli*.

Methylation of position 5 in cytidines is another common rRNA modification (23), among others found around helix 69/71 in eubacteria and eukaryotes. No general function has been ascribed to 5-methylcytidine (m\(^5\)C) in rRNA, but inactivation of the enzyme methylating C1962 in *E. coli* 23 S rRNA results in slightly reduced growth (24).

X-ray crystallographic structures have been reported for the m\(^5\)C methyltransferase RmI, which introduces m\(^5\)C1962 in 23 S rRNA (25, 26), and for m\(^5\)C methyltransferases of the small ribosomal subunit (RsmB and RsmF, which modify C967 and C1407, respectively (27–29)). No substrate complex structures

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‡ This article contains supplemental Figs. S1 and S2.

1 The atomic coordinates and structure factors (code 4DMG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

2 To whom correspondence may be addressed: Dept. of Molecular Biology, Cell Biology and Biochemistry, Brown University, Box G-E129, Providence, RI 02912. Tel.: 401-863-6123; Fax: 401-863-6114; E-mail: Gerwald_Jogl@brown.edu.

3 The abbreviations used are: used: m\(^5\)C, 5-methylcytidine; AdoMet, S-adenosyl-L-methionine; LC-MS, Liquid chromatography tandem mass spectrometry; r.m.s., root mean square.
for m^5C methyltransferases have been determined to date. However, structural studies of the functionally related m^5U methyltransferases RlmB (30) and TrmA (31) in complex with substrate oligonucleotides have been reported.

In the present work, we identify the 5-methylcytidine modifications of *Thermus thermophilus* 23 S rRNA at positions 1942 and 1962 and the responsible enzymes. Furthermore, a high resolution x-ray crystallography structure of TTHA1493, which 5-methylates C1942, is reported.

**EXPERIMENTAL PROCEDURES**

*Cloning of the T. thermophilus TTHA1280- and TTHA1493-encoding Genes*—The *T. thermophilus* HB8 loci encoding TTHA1280 and TTHA1493 were PCR-amplified from genomic DNA based on the genome sequence (GenBank™ accession number NC_006461.1). General information on the cloning has been reported in Ref. 29; gene-specific details were as follows. The PCR primers for TTHA1280 amplifications were CTTAGCATGAGGATGGAGTC and GCCCTCG- GATCTGAGGACC with conditions for PCR as in Ref. 29. The TTHA1493 gene was PCR-amplified in a two-step nested primer approach with the first set of primers having the sequences CCGAGGAAGAGGGCGAGGGC and CCTGAGGATCTGAGGACC with conditions for PCR as in Ref. 29.

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**Purification of Ribosomes, Ribosomal Subunits, rRNA, and 23 S rRNA Subfragments**—These purifications were in most instances done as described previously (32). The DNA oligonucleotides for purification of 23 S rRNA subfragments were CCGTTATAGTTACGGCC and GCGCTCCGGTCGTTACGGAACTTACCCGACAAGGAATTTCGCTACCTTAGGA, which were complementary to positions 1906–1958 and 1947–1990 (E. coli numbering throughout), respectively, of *T. thermophilus* 23 S rRNA.

For the nucleoside analyses, the following procedure for purification of *T. thermophilus* total RNA was used. 200 ml of an overnight culture of *T. thermophilus* HB8 in *Thermus* medium (2 g/liter NaCl, 4 g/liter yeast extract, 8 g/liter Poly-peptone; adjusted to pH 7.5 with NaOH) was cooled on ice and harvested by centrifugation at 6000 rpm/4 °C for 10 min with a Beckman JA14 rotor. The cell pellet was washed with 50 ml of Buffer A (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM NH₄Cl, 100 mM KCl), centrifuged again, and resuspended in 8 ml of Buffer A. The cells were lysed by sonicaton (8 × 30 s with 30 s on ice in between), and cell debris was removed by 2× centrifugation at 16,000 rpm/4 °C for 10 min with a microcentrifuge. The supernatant was adjusted to 0.5% SDS, 40 mM Tris, pH 8.0, and 10 mM EDTA followed by the addition of 160 mg of protease K and incubation at 37 °C for 1 h. The aqueous phase was extracted five times with phenol, two times with phenol/ chloroform, and once with chloroform. The RNA was precipitated with 1/10 volume of 3 M sodium acetate/3 volumes of 96% ethanol, washed with 70% ethanol, and redissolved in 220 μl of water. 23 S rRNA subfragments were isolated directly from the total RNA preparation.

**Digestion of 23 S rRNA Subfragments and Mass Spectrometry**—RNase A and RNase T1 digestions followed by MALDI-TOF mass spectrometric analysis were done as described previously (33). Digestion of rRNA into nucleosides and subsequent nanoChip liquid chromatography-tandem mass spectrometry (LC-MS') was done according to Giessing et al. (34). Nucleosides were base-linearly separated on a graphitic carbon chip under reduced conditions and identified by automatic tandem mass spectrometry (up to MS³) in real time.

**In Vitro Methylation**—Reactions contained 50 pmol of 23 S rRNA, 50 S subunits, or 70 S ribosomes from the *T. thermophilus* ΔTTHA1493 mutant as substrate in a total volume of 100 μl containing 100 mM NH₄Cl, 10 mM MgCl₂, 40 mM Hepes (pH 7.5), 6 mM β-mercaptoethanol and 10% glycerol (prepared as a 2× concentrated stock solution), 1.5 mM S-adenosyl-L-methionine (AdoMet), and 2 µg of recombinantly expressed TTHA1493 (see below). Water and stock buffer were mixed and left at room temperature for 15 min. Then substrate, enzyme, and AdoMet were added and incubated at 70 °C for 1 h. Reactions were stopped by phenol/chloroform extraction, and the RNA was recovered by ethanol precipitation before purification of the 23 S rRNA subfragment with the oligonucleotide CGTGCAGGTCGAATTACCGACAAGGATTTTCCGCTACTTATGGAG- CGGTATAGTTACGGCC and GCCCTCGGCTGTCGTTACGGAACTTACCCG, which were complementary to positions 1919–1967 of the 23 S rRNA) as described above. Control reactions without enzyme or AdoMet were carried out in all instances.

**Growth Competition Assay**—Details can be found in a previous study (29). In brief, wild type and ΔTTHA1493 *T. thermophilus* were co-cultured at 60, 70, or 80 °C in *Thermus* medium (see above). After growth for 24 h, each culture was transferred to fresh 5 ml of medium and incubated at the respective temperatures for another 24 h. This was repeated in independent triplicates for seven cycles. Samples were collected at each dilution; half was spread on plates without antibiotic, and the other half was spread on plates with 30 μg/mL kanamycin. Plates were incubated at 70 °C until colonies became visible.
**Protein Expression, Purification, and Crystallization—**

*E. coli* BL21 (DE3) (Invitrogen) containing pLJ102-TTHA1493 was grown to mid-log phase in LB medium at 37 °C in the presence of 100 μg/ml ampicillin. Protein expression was induced at 20 °C with 400 μM isopropyl-1-thio-β-D-galactopyranoside. Cells were pelleted after 18 h by centrifugation at 4000 rpm for 20 min at 4 °C and lysed by ultrasonication on ice in a buffer containing 20 mM Tris-HCl (pH 8.5), 100 mM NaCl, 5 mM β-mercaptoethanol, 0.1% Triton X-100, and 5% glycerol. Cell debris and membranes were pelleted by centrifugation at 14,000 rpm for 30 min at 4 °C. Soluble C-terminally hexahistidine-tagged *T. thermophilus* TTHA1493 was further purified by affinity chromatography with nickel-nitrilotriacetic acid resin (Qiagen). Untagged proteins were removed with washing buffer containing 20 mM Tris-HCl (pH 8.5), 250 mM NaCl, and 10 mM imidazole (pH 8.5). Recombinant TTHA1493 was then eluted with buffer containing 20 mM Tris-HCl (pH 8.5), 250 mM NaCl, and 150 mM imidazole. The protein was then purified by anion exchange chromatography at pH 8.5, using a linear gradient of 10 mM to 1 M NaCl. TTHA1493 fractions were pooled and concentrated and applied to a size-exclusion S200 column of 10 mM to 1 M NaCl. TTHA1493 fractions were pooled and concentrated and applied to a size-exclusion S200 column of 10 mM to 1 M NaCl. TTHA1493 fractions were pooled and concentrated and applied to a size-exclusion S200 column of 10 mM to 1 M NaCl. TTHA1493 fractions were pooled and concentrated and applied to a size-exclusion S200 column of 10 mM to 1 M NaCl.

**Data Collection, Structure Determination, and Refinement—**

X-ray diffraction data to 1.7 Å resolution were collected on an X4A beamline of the National Synchrotron Light Source in the Brookhaven National Laboratory at a wavelength of 0.979 Å and −180 °C. Crystals belong to space group P2₁ with cell dimensions a = 63.8 Å, b = 46.0 Å, c = 129.4 Å, and β = 96.8 Å. Diffraction images were integrated and scaled with the HKL2000 package (35). The data processing statistics are summarized in Table 1. The structure was solved by molecular replacement with the program BALBES (36) from the CCP4 package (37) using the structure of the PH1915 methyltransferase from *Pyrococcus horikoshii* (38). The initial structure solution was then rebuilt with the program ARP/wARP (39) and manually completed with the program Coot (40). TLS domains were identified using the program TLSMD (41), and refinement was performed using Phenix (42). There are two molecules in the asymmetric unit. The crystallographic R and Rfree factors are 0.179 and 0.208. The quality of the final model was evaluated with MolProbity (43). The Ramachandran statistics (most favored/additionally allowed/disallowed) are 98/2/0.0%. Refinement statistics are summarized in Table 1. Figures were generated using PyMOL (50).

**RESULTS**

Identification of the *T. thermophilus* 23 S rRNA m⁵C1942 and m⁵C1962 Modifications and of the Corresponding Methyltransferases—

We previously showed that C1942 and C1962 (C1963 and C1983 in *T. thermophilus*) of 23 S rRNA in *T. thermophilus* are singly methylated (16), but did not determine the precise location of the methyl groups on the two nucleotides. To clarify these points, we isolated the modified cytidines separately by purification of two 23 S rRNA subfragments (positions 1906–1958 and positions 1947–1990, respectively) followed by total hydrolysis into nucleosides. The nucleosides were subjected to LC-MS² on an ion trap mass spectrometer, and the identity of the methylated cytidines was determined from a combination of the chromatographic retention time on a graphite column combined with the MS² fragmentation pattern (34). Fig. 1 demonstrates that each of the 23 S RNA subfragments harbors cytidines methylated at carbon-5 of the nucleobase, where the retention time and mass spectrometric fragmentation pattern (not shown) are identical to that of an m⁵C standard. Our analysis additionally confirmed a 2′-O-ribose cytidine methylation (Cm), which we have previously mapped to C1920. Hence, C1942 and C1962 are 5-methylated.

A 5-methyl modification of C1962 is also found in *E. coli* 23 S rRNA, synthesized by the enzyme RlmI (24). We used the sequence of the *E. coli* RlmI protein to perform a BLAST search against all potential *T. thermophilus* proteins to obtain candidates for methyl transferases that target C1942 and C1962. Our working hypothesis was that m⁵C methyltransferases, which target nucleotides that are located close to each other in both secondary and three-dimensional conformation (see Fig. 6D), and that they will exhibit significant sequence homology because they likely recognize similar RNA structures. The search returned the hypothetical protein TTHA1280 as the top
T. thermophilus m^5C1942 Methyltransferase

![Experimental data](image)

**FIGURE 2.** MALDI-TOF mass spectrometry of RNase T1-digested T. thermophilus 23 S rRNA positions 1906–1958; zoom is on the m/z region where the digestion product positions 1936–1945 are located. *Top*, from strain with disrupted TTHA1493 gene. *Middle*, from strain with disrupted TTHA1280 gene. *Bottom*, from wild type strain.

match with an expected value of $10^{-61}$, followed by TTHA1493 with an expected value of $10^{-46}$.

The genes encoding the two proteins were cloned into an expression vector (44) and also used to construct gene knockouts in *T. thermophilus* through insertion of a kanamycin resistance cassette (45). 23 S rRNA subfragments encompassing C1942 or C1962 were purified from the two *T. thermophilus* knock-out strains. The rRNA subfragments were digested with either RNase A or RNase T1, and digestion products were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Fig. 2 shows that ΔTTHA1493 gives a C1942-encompassing RNase T1 digestion product 14.0 Da less in mass than the wild type *T. thermophilus*. However, this RNase T1 digestion product also contains a methylated U1939 (16); i.e., it was a theoretical possibility that ΔTTHA1493 affected methylation of U1939 rather than C1942. To exclude this possibility, we performed nucleoside analysis of the ΔTTHA1493 rRNA subfragment harboring C1942 (Fig. 3). Here m^5C is absent (whereas Cm is still apparent), showing that inactivation of TTHA1493 prevents 5-methylation of C1942.

We previously showed that inactivation of the protein, which confers 5-methylation of C1400, C1404, and C1407 in 16 S rRNA of *T. thermophilus*, results in a dramatically reduced viability at non-optimal temperatures (29). We likewise compared the temperature sensitivity of the ΔTTHA1493 strain with that of the wild type, but observed no growth difference at 60, 70, or 80 °C (data not shown).

Supplemental Fig. S1 reveals that the ΔTTHA1280 strain, but not the ΔTTHA1493 strain, completely lacks the methyl group on the RNase A digestion product harboring C1962. This is in line with *a priori* expectations based on the outcome of the above BLAST search using *E. coli* RlmI and strongly suggests that TTHA1280 is also a functional homolog of *E. coli* RlmI. Because the structures of TTHA1280 (26) and RlmI (25) have already been reported, we did not pursue investigations on this protein.

**Substrate Preference**—*In vitro* methylation assays were set up to explore the substrate of TTHA1493, but also to ascertain that C1942 methylation is directly catalyzed by this protein. 70 S ribosomes, 50 S ribosomal subunits, and 23 S rRNA from the ΔTTHA1493 strain were prepared via sucrose gradient centrifugation, where all ribosomal components exhibited a normal behavior. The 70 S ribosome, 50 S ribosomal subunit, or 23 S rRNA was used as substrates, and AdoMet was the methyl group donor in reactions with purified TTHA1493 protein. The outcome of *in vitro* methylation of 23 S rRNA is shown in Fig. 4, where an essentially complete methylation of the C1942-containing RNase T1 digestion product is revealed. This means that 23 S rRNA is an efficient substrate for TTHA1493, whereas no methylation of either 50 S ribosomal subunits or 70 S ribosomes could be obtained *in vitro* (data not shown). Hence, TTHA1493 is a genuine AdoMet-dependent 23 S rRNA methyltransferase, and we propose that it is named RlmO in accordance with the nomenclature previously suggested (44).

**X-ray Crystal Structure of TTHA1493**—We determined the structure of *T. thermophilus* TTHA1493 (385 amino acids) in complex with AdoMet cofactor to 1.7 Å resolution. The structure was solved by molecular replacement with the program BALBES (36) from the CCP4 package (37) using the structure of the PH1915 methyltransferase from *P. horikoshii* (38). There are two molecules in the asymmetric unit, although RlmO appears to be monomeric in solution as estimated from size-exclusion chromatography (data not shown). Electron density was generally well defined for both chains and the cofactor molecules. The density for the cofactor methyl group was weaker, indicating partial conversion to S-adenosyl-homocysteine. The final models consist of residues 1–385 in both chains. No clear electron density was observed for the m^5C methyl group donor in reactions with purified TTHA1493 protein. The structure of the PH1915 methyltransferase from *P. horikoshii* (38) is available in the Protein Data Bank (PDB) under the identifier 3PH1. The overall structure of TTHA1493 consists of two N-terminal domains, a β-hairpin and short linker helix, and a canonical class I methyltransferase catalytic domain (Fig. 5A). The first N-terminal domain (residues 6–67) contains a four-stranded β-sheet and one stabilizing helix and is related to PUA domains (46). The central domain consists of a five-stranded β-sheet and three helices (residues 75–170). The catalytic domain is formed...
by a central seven-stranded β-sheet that is supported by three helices on each side (residues 198–385). The AdoMet cofactor is bound in a canonical position in a pocket of the catalytic domain (Fig. 5B). The adenine base is positioned by hydrophobic contacts to Tyr-222 and the aliphatic carbon chain of Lys-244. The ribose hydroxyl groups are positioned by hydrophobic contacts to Tyr-222 and the aliphatic carbon chain of Lys-244. The ribose hydroxyl groups are coordinated by hydrogen bonds to Arg-205, Asp-287, and the backbone carbonyl of Tyr-222. Tyr-198 defines the pocket of the catalytic domain (Fig. 5D). In monomer A, the side chains of Thr-290 and Lys-293 are rotated toward the active site in a potential RNA binding conformation. Both residues are oriented away from the active site in molecule B, where Leu-291 assumes the position of Lys-293 in the first monomer. However, both conformations of this region are unlikely to represent the final substrate-bound state as both active site conformations are too narrow to accommodate a substrate cytidine without further adjustments.

**DISCUSSION**

**Structural Comparison with Related Methyltransferases**—A database search with Dali (47) confirmed that the overall structure of the RlmO methyltransferases from *E. coli* (Protein Data Bank (PDB) entry 3C0K (25)) and *T. thermophilus* (PDB entry 1WXW (26)) and the PH1915 methyltransferase (PDB entry 2AS0 (38)) are the most similar structures in the Protein Data Bank (PDB) entry 3C0K (25)). A comparison of the two RlmO monomers in the asymmetric unit reveals several regions with differing conformations (Fig. 5C). The two monomers can be superimposed with an overall root mean square (r.m.s.) deviation of 0.75 Å for all 385 Ca atoms. All variable regions are located in the catalytic domain near the active site. This suggests that the binding of substrate RNA to RlmO utilizes an induced fit mechanism. Inspection of one of the flexible regions between residues 288 and 299 in the RlmO active site reveals a substantial rearrangement between potential hydrogen bond donors and hydrophobic residues (Fig. 5D). In monomer A, the side chains of Thr-290 and Lys-293 are rotated toward the active site in a potential RNA binding conformation. Both residues are oriented away from the active site in molecule B, where Leu-291 assumes the position of Lys-293 in the first monomer. However, both conformations of this region are unlikely to represent the final substrate-bound state as both active site conformations are too narrow to accommodate a substrate cytidine without further adjustments.
Asp-200 in RlmO are conserved, as are most of the cofactor-
23 S helix 71 (colored in blue, PDB entry 2BH2 (30)). The covalently bound substrate base U1939 is shown colored in magenta. D, location of modified bases in the 23 S rRNA region with rRNA modifications and names of corresponding methyltransferases.

RlmI. However, how this homodimeric surface binds substrate
methyltransferase functions as a homodimer as proposed for
RlmI and RlmO enzymes and the continuous positively charged
interface in the dimer suggests that the RlmO active sites above. The close-up view of the active site precludes computational docking of the substrate binding. RlmD substrate complex structure, the position of the target U1939 is stabilized by hydrophobic contacts to Phe-263. Tyr-198 occupies this position in RlmO to provide the same potential stabilization for substrate placement. RlmD residue Glu-265 was shown to be critical for recognition of the substrate uracil. This residue is not conserved in the m^5C methyltransferase RlmO. Instead, Asp-200 approaches an approximately equivalent position to potentially provide hydrogen bonds to a substrate cytosine. A notable difference between the two structures is the absence of an RlmO residue equivalent to Glu-424. This residue acts as the general base in RlmD to resolve the covalent enzyme-substrate intermediate after methyl group transfer. Considering likely small differences in substrate base orientation between m^5U and m^5C methyltransferases, Asp-200 could potentially fulfill this critical function.

**Potential Function of the Modification**—C1942 is surface-exposed in the 50 S ribosomal subunit (48), but is close to the A-site tRNA in the complete ribosome (Fig. 6D). The nucleotide is stacked between C1941 and U1943, which again are part of a longer stacking series. The position 5 methyl group points away from the subunit surface and the A-site tRNA and is located so that it likely strengthens the stacking interactions with 23 S rRNA residues 1941 and 1943. However, experiments are required to delineate the precise effect of C1942 methylation in the local structure.

Modification of C1942 in the large ribosomal subunit RNA is not a common phenomenon; it has, to the best of our knowledge, been suggested only in *Arabidopsis thaliana*, where a sequence-specific small nucleolar RNA hypothetically guiding C1942 ribose methylation has been found (49). In an independent work, we have also identified m^5C1942 in *Deinococcus
radiodurans.4 A BLAST search using TTHA1493 as query accordingly revealed homologues with the highest similarity in the genus Deinococcus, but the class Fusobacteria also had TTHA1493 homologues with an expected value similar to the one in D. radiodurans (around 10−70 covering the entire sequence). Hence, we would expect the presence of m5C1942 in 23S rRNA from multiple other species.

We have previously shown that inactivation of the protein TTHA1387, which confers 5-methyltransferase of C1400, C1404, and C1407 in 16S rRNA of T. thermophilus, results in a dramatically reduced viability both above and below 70 °C (29), whereas the ΔTTHA1493 strain does not exhibit such a phenotype. TTHA1387 has homologues primarily in thermophilic bacteria, indicating that this enzyme and the conferred rRNA methylations are involved in thermal adaption. TTHA1493 homologues are not confined to thermophiles, in concurrence with the lack of significance of the protein in thermal tolerance.

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REFERENCES

1. Sirum-Connolly, K., Peltier, J. M., Crain, P. F., McCloskey, J. A., and Mason, T. L. (1995) Implications of a functional large ribosomal RNA with only three modified nucleotides. Biochimica 77, 30–39
2. Cantara, W. A., Crain, P. F., Rozenjsi, J., McCloskey, J. A., Harris, K. A., Zhang, X., Vendeix, F. A., Fabris, D., and Agris, P. F. (2011) The RNA Modification Database, RNAMDB: 2011 update. Nucleic Acids Res. 39, D195–D201
3. Havelund, J. F., Giessing, A. M., Hansen, T., Rasmussen, A., Scott, L. G., and Kirpekar, F. (2011) Identification of 5-hydroxycytidine at position 2501 concludes characterization of modified nucleotides in E. coli 23S rRNA. J. Mol. Biol. 411, 529–536
4. Maden, B. E. (1988) Locations of methyl groups in 28S rRNA of Xenopus laevis and man: clustering in the conserved core of molecule. J. Mol. Biol. 201, 289–314
5. Maden, B. E. (1990) The numerous modified nucleotides in eukaryotic ribosomal RNA. Prog. Nucleic Acid Res. Mol. Biol. 39, 241–303
6. Ofengand, J., and Bakin, A. (1997) Mapping to nucleotide resolution of pseudouridine residues in large subunit ribosomal RNAs from representative eukaryotes, prokaryotes, archaeabacteria, mitochondria, and chloroplasts. J. Mol. Biol. 266, 246–268
7. Rauë, H. A., Kloetwijk, J., and Masters, W. (1988) Evolutionary conservation of structure and function of high molecular weight ribosomal RNA. Prog. Biophys. Mol. Biol. 51, 77–129
8. Cavallé, J., Nicoloso, M., and Bachelerie, J. P. (1996) Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. Nature 383, 732–735
9. Ganot, P., Bortolin, M. L., and Kiss, T. (1997) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell 79, 799–809
10. Kiss-László, Z., Henry, Y., Bachelerie, J. P., Caizergues-Ferrer, M., and Kiss, T. (1996) Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. Cell 85, 1077–1088
11. Tycowski, K. T., Smith, C. M., Shu, M. D., and Steitz, J. A. (1996) A small nucleolar RNA requirement for site-specific ribose methylation of RNA

4 T. Hansen and F. Kirpekar, unpublished data.
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1584 – 1596

30. Lee, T. T., Agarwalla, S., and Stroud, R. M. (2005) A unique RNA fold in the RumA-RNA-cofactor ternary complex contributes to substrate selectivity and enzymatic function. Cell 120, 599 – 611

31. Alian, A., Lee, T. T., Griner, S. L., Stroud, R. M., and Finer-Moore, J. (2008) Structure of a TrmA-RNA complex: a consensus RNA fold contributes to substrate selectivity and catalysis in m5U methyltransferases. Proc. Natl. Acad. Sci. U.S.A. 105, 6876 – 6881

32. Andersen, T. E., Porse, B. T., and Kirpekar, F. (2004) A novel partial modification at C2501 in Escherichia coli 23 S ribosomal RNA. RNA 10, 907 – 913

33. Douthwaite, S., and Kirpekar, F. (2007) Identifying modifications in RNA by MALDI mass spectrometry. Methods Enzymol. 425, 1 – 20

34. Giessing, A. M., Scott, L. G., and Kirpekar, F. (2011) A nano-Chip-LC/MS\textsuperscript{n} based strategy for characterization of modified ribonucleic acids using reduced porous graphitic carbon as a stationary phase. J. Am. Soc. Mass. Spectrom. 22, 1242 – 1251

35. Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307 – 326

36. Long, F., Vagin, A. A., Young, P., and Murshudov, G. N. (2008) BALBES: a molecular replacement pipeline. Acta Crystallogr. D. Biol. Crystallogr. 64, 125 – 132

37. Collaborative Computational Project, Number 4 (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D. Biol. Crystallogr. 50, 760 – 763

38. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D. Biol. Crystallogr. 66, 12 – 21

39. Langer, G., Cohen, S. X., Lamzin, V. S., and Perrakis, A. (2008) Automated macromolecular model building for X-ray crystallography using ARP/wARP version 7. Nat. Protoc. 3, 1171 – 1179

40. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D. Biol. Crystallogr. 60, 2126 – 2132

41. Painter, J., and Merritt, E. A. (2006) Optimal description of a protein structure in terms of multiple groups undergoing TLS motion. Acta Crystallogr. D. Biol. Crystallogr. 62, 439 – 450

42. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstele, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D. Biol. Crystallogr. 66, 213 – 221

43. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D. Biol. Crystallogr. 66, 12 – 21

44. Andersen, N. M., and Douthwaite, S. (2006) YebU is a m5C methyltransferase specific for 16 S rRNA nucleotide 1407. J. Mol. Biol. 359, 777 – 786

45. Hashimoto, Y., Yano, T., Kuramitsu, S., and Kagamiyama, H. (2001) Disruption of Thermus thermophilus genes by homologous recombination using a thermostable kanamycin-resistant marker. FEBS Lett. 506, 231 – 234

46. Pérez-Arellano, I., Gallego, J., and Cervera, J. (2007) The PUA domain: a structural and functional overview. FEBS J. 274, 4972 – 4984

47. Holm, L., Kääriäinen, S., Rosenström, P., and Schenkel, A. (2008) Searching protein structure databases with DALI Lite v.3. Bioinformatics 24, 2780 – 2781

48. Stanley, R. E., Blaha, G., Grodzicki, R. L., Strickler, M. D., and Steitz, T. A. (2010) The structures of the anti-tuberculosis antibiotics viomycin and capreomycin bound to the 70 S ribosome. Nat. Struct. Mol. Biol. 17, 289 – 293

49. Barneche, F., Gaspin, C., Guyot, R., and Echeverría, M. (2001) Identification of 66 box C/D snoRNAs in Arabidopsis thaliana: extensive gene duplications generated multiple isoforms predicting new ribosomal RNA 2′-O-methylation sites. J. Mol. Biol. 311, 57 – 73

50. DeLano, W. L. (2010) The PyMOL Molecular Graphics System, version 1.3r1, Schrödinger, LLC, New York