Characterization and Structure of the *Aquifex aeolicus* Protein DUF752

**A BACTERIAL tRNA-METHYLTRANSFERASE (MnmC2) FUNCTIONING WITHOUT THE USUALLY FUSED OXIDASE DOMAIN (MnmC1)**

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**Background:** *Escherichia coli* encodes a bifunctional oxidase/methyltransferase catalyzing the final steps of methylaminomethyluridine (mnmU) formation in tRNA wobble positions.

**Results:** *Aquifex aeolicus* encodes only a monofunctional aminomethyluridine-dependent methyltransferase, lacking the oxidase domain.

**Conclusion:** An alternative pathway exists for mnmU biogenesis.

**Significance:** Information about how an organism modifies the wobble base of its tRNA is important for understanding the emergence of the genetic code.

Post-transcriptional modifications of the wobble uridine (U34) of tRNAs play a critical role in reading NNA/G codons belonging to split codon boxes. In a subset of *Escherichia coli* tRNA, this wobble uridine is modified to 5-methylaminomethyluridine (mnmU) through sequential enzymatic reactions. Uridine 34 is first converted to 5-carboxymethylaminomethyluridine (cmnmU) by the MnmE-MnmG enzyme complex. The cmnmU is further modified to mnmU by the bifunctional MnmC protein. In the first reaction, the FAD-dependent oxidase domain (MnmC1) converts cmnmU into 5-aminomethyluridine (nm5U) by the MnmC protein. In the second reaction, the nm5U is immediately followed by the methylation of the free amino group into mnmU by the MnmC2 domain. Here, we determined the crystal structure of the *A. aeolicus* DUF752 protein at 2.5 Å resolution, which revealed that it catalyzes the S-adenosylmethionine-dependent methylation of nm5U into mnmU in vitro, to form mnmU34 in tRNA. We also showed that naturally occurring tRNA from *A. aeolicus* contains the 5-mnm group attached to the C5 atom of U34. Taken together, these results support the recent proposal of an alternative MnmC1-independent shortcut pathway for producing mnmU34 in tRNAs.

Transfer RNAs (tRNAs) contain a wide variety of post-transcriptionally modified nucleosides. Among them, the wobble nucleoside at position 34 of the tRNA anticodon is the most diversely modified one identified so far in a naturally occurring cellular tRNA (1). The types of chemical adducts existing on the various atoms of nucleoside 34 strongly depend on the isoacceptor tRNA and the organism from which the nucleic acid originated (2, 3). For example, in *Escherichia coli* tRNA^Glu^, tRNA^Asp^, and tRNA^Glu^, the wobble uridine 34 is hypermodified into a 5-methylaminomethyl-2-thiouridine (mnmS^2^U), whereas in tRNA^Asp^ and tRNA^Glu^, only a 5-mnm group is found on the nonthiolated U34 (mnmU). In one of the two *E. coli* U34-containing tRNA^Glu^ molecules, the C5 atom of U34 is modified into a cmnm group, and its 2′-hydroxyl ribose is methylelated into U, leading to the doubly hypermodified cmnmUm34 residue. In *Bacillus subtilis* and *Mycoplasma*
capricolum, only cmm\(^{5}(s^{2})\)U(m)-type derivatives are found in the split codon box tRNAs (2, 4, 5), although in the majority of other bacteria the chemical identity of the wobble U34 is often not known. Finally, mammalian mitochondrial tRNAs contain a taurine derivative (cmm\(^{5}(s^{2})\)U(m)) (6, 7). These various derivatives of the wobble nucleoside are collectively designated as Xm\(^{5}(s^{2})\)U(m), where X corresponds to distinct adducts of the minimal 5-methyluridine 34. Together with other modified nucleosides in the anticodon branch, they allow the modified tRNA to read selected mRNA codons more efficiently and accurately during translation on the ribosome (8–11).

Several enzymes catalyze the formation of Xm\(^{5}\)U34 derivatives in *E. coli*. These include the GTPase MnmE (formerly designated as TrmE), the folate-dependent MnmG (GidA), and the bifunctional oxidase methyltransferase MnmC. The 2-thiolation is catalyzed by the thiolase MnmA, and the methyltransferase TrmL mediates the 2\(^{\text{th}}\)-hydroxyl methylation of U34, regardless of whether the C5 atom of U34 is modified (12). Two alternative mechanisms have been proposed for the multistep enzymatic formation of cmm\(^{5}\)U34 in tRNA (Fig. 1A). In the first pathway, the formation of 5-carboxymethylaminomethyluridine is catalyzed by MnmE and MnmG, using glycine, a methyl derivative of tetrahydrofolate, GTP, and FAD as cofactors. This complex reaction has been recapitulated under in vitro conditions, using purified recombinant MnmE-MnmG enzymes. However, its detailed mechanism is still a matter of debate (13–18). In the last two enzymatic steps, the 5-cmm adduct is converted to the 5-cmm derivative (cmm\(^{5}\)U34) by the bifunctional protein MnmC (18–22). The C-terminal domain of this protein (designated as MnmC1) first catalyzes the transformation of the 5-cmm adduct of U34 into a 5-aminomethyluridine intermediate (cmm\(^{5}\)U34), which is subsequently methylated to cmm\(^{5}\)U34 by the methyltransferase of the N-terminal domain (DUF752, designated as MnmC2), using AdoMet as the methyl donor (Fig. 1A, upper part). The cmm\(^{5}\)U34-containing tRNA intermediate usually does not accumulate in normal *E. coli* cells, except in certain mutants such as rel\(^{-}\), met\(^{-}\), or those defective in the MnmC2 activity (19, 20, 23, 24). In the second alternative “shortcut” pathway, recently discovered by Armengod and co-workers (17), ammonium, instead of glycine, is used by the MnmE-MnmG complex to catalyze the direct formation of cmm\(^{5}\)U34-containing tRNA, thus bypassing the need for the MnmC1 activity (Fig. 1A, lower part). This cmm\(^{5}\)U34-containing tRNA intermediate is further methylated into the same final cmm\(^{5}\)U34 product as above by the MnmC2 domain of the bifunctional MnmC. This cmm\(^{5}\)U34-to-cmm\(^{5}\)U methylation reaction has been demonstrated to occur in vitro, using a purified recombinant MnmC enzyme defective in the MnmC1 activity, thus showing that each of the two enzymatic activities of the fusion MnmC protein can be uncoupled and work independently in trans-complementation types of experiments (21, 22, 24).

These observations prompted us to examine the situation in *Aquifex aeolicus*. This bacterial species has genes encoding the MnmE, MnmG, and MnmA but only has one gene encoding a shorter version of MnmC (DUF752). This gene appears to be the homolog of MnmC2, and it seems to lack the FAD oxidase (DAO)-like MnmC1 domain (Fig. 1B). In this study, we first analyzed the *A. aeolicus* tRNAs and confirmed the presence of cmm\(^{5}\)U34-containing tRNA species. We next showed that purified *A. aeolicus* monofunctional DUF752 binds tRNA and displays the expected methyltransferase activity in vitro. Finally, we determined the crystal structure of *A. aeolicus* DUF752, which revealed the interesting tRNA-binding characteristics of this newly identified bacterial methyltransferase.
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EXPERIMENTAL PROCEDURES

Strains and Isolation of Bulk tRNA from A. aeolicus and B. subtilis—Bulk tRNA from B. subtilis strain 168 (wild type) was obtained as described previously (25). Bulk tRNA from A. aeolicus VF5 was obtained by the same procedure.

Protein Expression and Purification—The gene encoding the DUF752 protein (aq_1980, gi:15606976), corresponding to the putative methyltransferase MnmC2, was amplified via PCR using A. aeolicus VF5 genomic DNA and was cloned into the pET-11b expression vector (Merck Novagen). The expression vector was introduced into the E. coli BL-21(DE3) strain (Merck Novagen), and the recombinant strain was cultured in 2.5 liters of LB medium. The harvested cells were resuspended in 20 mM Tris-Cl buffer (pH 8.0), containing 300 mM NaCl, 5 mM MgCl₂, and 2 mM DTT, and subsequently lysed by sonication four times for 30 s on ice (VP-30, TAITEC). After centrifugation at 27,000 x g for 30 min (CR22GIII, Hitachi), the supernatants were heat-treated at 70 °C for 30 min and purified by a series of HiTrap-phenyl, HiTrap-Q, and Resource-S (GE Healthcare) column chromatography steps, followed by dialysis against 20 mM Tris-Cl buffer (pH 8.0), containing 150 mM NaCl and 2 mM DTT. The protein sample was then purified by gel filtration on a Superdex 75 10/300GL column (GE Healthcare) and was concentrated by centrifugal filtration (Millipore) to 11.3 mg/ml.

For the x-ray crystallization experiment, the selenomethionine-labeled (SeMet) A. aeolicus DUF752 protein was obtained by expression from the same pET-11b vector (Merck Novagen) bearing the tag sequence at the N terminus (26), and the protein was purified in the same manner as the native proteins. The purified protein was then biotinylated by an incubation with E. coli biotin ligase, BirA, and biotin at 37 °C for 2 h.

The gene encoding MnmG (aq_761, gi:15606146) from A. aeolicus VF5 was cloned into pET-15b (Merck Novagen) and expressed in the E. coli Rosetta(DE3) strain (Merck Novagen). The harvested cells were resuspended in 50 mM HEPES buffer (pH 7.0), containing 500 mM NaCl, 500 mM MgCl₂, and 2 mM DTT. After sonication for four times for 30 s (VP-30, TAITEC) and centrifugation at 15,000 x g for 30 min (CR22GIII, Hitachi), the supernatants were heat-treated at 75 °C for 15 min and purified by a series of HiTrap-Q, HiTrap-heparin, and Resource-Q (GE Healthcare) column chromatography steps. The sample buffer was exchanged by dialysis at 4 °C to 20 mM Tris-Cl buffer (pH 8.0), containing 300 mM NaCl and 2 mM DTT. The protein sample was then purified by gel filtration on a Superdex 75 10/300GL column (GE Healthcare) and was concentrated by centrifugal filtration (Millipore) to 7.6 mg/ml.

The gene encoding MnmC (JW5380, gi:85675355) from E. coli was cloned into pET-15b (Merck Novagen) and expressed in the E. coli Rosetta2(DE3) strain (Merck Novagen). The expression vector containing MnmC, bearing a mutation in the essential Asp-178 (D178A), was prepared using a QuickChange™ site-directed mutagenesis kit (Stratagene) and expressed in the same E. coli Rosetta2(DE3) strain (Merck Novagen). The purification procedures for these recombinant proteins were described previously (27).

Crystallization and Data Collection—For the crystallization assay, the purified A. aeolicus DUF752 protein samples were first concentrated to 13–26 mg/ml in 20 mM Tris-Cl buffer (pH 8.0), containing 150 mM NaCl and 2 mM DTT. The SeMet DUF752 protein was co-crystallized with 1 mM AdoMet by the hanging-drop vapor diffusion method. Preliminary screenings were performed using the Hampton Research Index Screen kit, and small crystals appeared with a reservoir solution consisting of 0.1 M sodium acetate buffer (pH 4.5), containing 25%(w/v) PEG 3350. After optimization, large crystals were obtained, using a reservoir solution of 0.1 M sodium acetate buffer (pH 5.0), containing 25%(w/v) PEG3350 and 5%(w/v) benzamidine. Rectangular parallel-piped crystals grew to dimensions of 0.2 x 0.1 x 0.1 mm at 20 °C in 10 days. For data collection, the crystals were flash-cooled in liquid nitrogen with 13% glycerol as a cryoprotectant. Diffraction data sets were collected at the BL41XU beamline at SPring-8 (Harima, Japan) and were processed by the use of the HKL2000 software suite.

Structure Determination and Refinement—The structure of the A. aeolicus DUF752 protein was determined by using the SeMet MAD data set, SOLVE and RESOLVE (28). We used the second data set (peak 2), obtained from another crystal in the same crystallization drop, to improve the electron density map. The model (2.5 Å) was built and refined with NCS restraints by using the programs Coot (29) and Refmac5 implemented in the CCP4 suite (30). Data collection and refinement statistics are summarized in Table 1. The quality of the protein model was inspected by PROCHECK (31). Structure representations were prepared with the PyMOL program (Schrödinger, LLC.). Coordinates and structure factors have been deposited in the Protein Data Bank, with the accession code 3VYW.

MTase Activity of A. aeolicus DUF752—All assays were performed at 45 °C in a 300-µl reaction mixture containing 40 mM Pipes (pH 6.4), 20 mM NH₄Cl, 0.2 mM EDTA, 0.2 mM DTT, 20 µM FAD, 4 µM S-adenosyl-L-[methyl-¹⁴C)methionine (2.22 GBq/mmol; GE Healthcare), 0.55 mg/ml (~20 µM) B. subtilis total tRNA (in which the C5 atom of several tRNAs harbors a
TABLE 1
Data collection, phasing, and refinement statistics

Values in parentheses are for highest resolution shell. r.m.s.d. means root mean square deviation.

| Peak | Edge       | Remote      | Peak 2     |
|------|------------|-------------|------------|
| Data set |            |             |            |
| X-ray source |             |             |            |
| Wavelength (Å) | 0.9792 | 0.9795 | 0.9792 |
| Resolution (Å) | 45–2.5 | 45–2.5 | 45–2.5 |
| Cell parameters |        |        |            |
| a (Å) | 55.42 | 55.42 | 55.42 |
| b (Å) | 108.04 | 108.07 | 107.58 |
| c (Å) | 117.76 | 117.79 | 117.71 |
| β | 102.19° | 102.18° | 102.44° |
| Space group | P2₁ | P2₁ |        |
| Molecules/asymmetric unit | 4 | 4 | 4 |
| Unique reflections | 47,010 | 46,996 | 47,040 |
| Redundancy | 42 | 42 | 41 |
| Completeness (%) | 99.9 (100%) | 99.9 (100%) | 99.8 (99.9%) |
| Figure of merit | 16.9 (2.7) | 17.4 (2.9) | 19.5 (3.9) |
| Phasing |            |             |            |
| Resolution (Å) | 20.0–2.5 | 20.0–2.5 | 20.0–2.5 |
| No. of sites | 8 | 8 | 8 |
| Figure of merit | 0.25 (after solvent modification, 0.57) | 0.25 (after solvent modification, 0.57) | 0.25 (after solvent modification, 0.57) |

Refinement

| Resolution range (Å) | 45.4–2.5 | 45.4–2.5 | 45.4–2.5 |
| B-factor/Rmerge (%) | 19.1/25.5 | 19.1/25.5 | 19.1/25.5 |
| No. of protein atoms | 10,068 | 10,068 | 10,068 |
| No. of hetero atoms | 126 | 126 | 126 |
| No. of water molecules | 146 | 146 | 146 |
| r.m.s.d. bond lengths (Å) | 0.028 | 0.028 | 0.028 |
| r.m.s.d. bond angles | 2.123° | 2.123° | 2.123° |
| Average B-value (Å²) | 50.89 | 50.89 | 50.89 |
| Ramachandran plot (%) | Core | Core | Core |
| Allowed | 94.1 | 94.1 | 94.1 |

α Rmerge = \[\Sigma F_{o} - \Sigma F_{c} \]/\[\Sigma F_{c}\],

β Rfree is the same as Rmerge but calculated using a small fraction (5%) of randomly selected reflections.

wobble cmnm\(^{(5)}U\)-U(m)34), 0.5 μM E. coli monofunctional MnmC-D178A mutant enzyme, and 5 μM A. aeolicus DUF752 protein. The E. coli mutant enzyme was used for producing the nm\(^{3}U\)-containing tRNA, which is the expected substrate of DUF752 (see under "Results"). At appropriate time points, 30-μl aliquots of the samples were mixed with a 20-fold volume of 10% cold trichloroacetic acid (TCA) and were incubated for 10 min on ice. The quenched samples were spotted on MF-Millipore membrane papers. The filters were washed five times with cold 10% TCA, and the radioactivity retained on the filter was measured with a liquid scintillation counter. The reactions were repeated five times, and the values were used to calculate the standard errors.

Enzyme-RNA Binding Assay—The interactions between the tRNA and the enzymes were analyzed with a Biacore 3000 SPR bio-sensor (GE Healthcare). The gene encoding the tRNAUUU from A. aeolicus (aq_t42, gene ID: 3284557) was amplified via PCR, using a 5'-primer including a T7 promoter and a corresponding synthetic nucleosides. The identification of m5s2U and cmnm5s2U was measured with a liquid scintillation counter. The reactions were repeated five times, and the values were used to calculate the standard errors.

Analysis of tRNA Modification by High Performance Liquid Chromatography—The purified tRNAs were digested to nucleosides, as described previously (32), and were analyzed by HPLC (Alliance, Waters) using a Develosil 5-μm RP-AQUEOUS C-30 reverse phase column (Phenomenex). The elution was monitored at 254 and 314 nm, and the gradient was employed as described previously (32, 33). The thiolytically cleaved nucleosides \(^{3}C\), \(^{5}C\), and \(^{5}C\) were identified by both their spectra and relative retention times to that of \(^{3}C\) and \(^{5}C\) were identified using the BIAevaluation 4.1 software package.

RESULTS

Comparison of Sequences and Domains—The amino acid sequence alignments of A. aeolicus DUF752 (upper line, 308 amino acids) and the N-terminal domains (254 amino acids) of the E. coli bifunctional MnmC methyltransferase (bottom line, 254 plus 414 amino acids) are shown in Fig. 2. A few additional homologous sequences from other organisms are also included between these two sequences (we also analyzed many others, data not shown). The high sequence similarity and the conservation of several key amino acids (nine altogether) suggested that both types of proteins possess similar and possibly identical functions. The sequence identity between A. aeolicus
DUF752 and *E. coli* MnmC2 is 26%, despite the fact that these two proteins are from species that grow at very different optimal temperatures (above 40 °C and optimally at 85–95 °C in the case of the thermophilic *A. aeolicus* and below 40 °C and optimally at 37 °C in the case of *E. coli*). The MnmC2 domains (N-terminal domains) of the bifunctional MnmC and DUF752 homologs both show the typical features of the AdoMet-dependent class I (Rossmann-fold) MTase family (motifs X and I–VIII, underlined in blue in Fig. 2) (34). The features include the partially conserved (GX)GXXG sequence within motif I, followed by a conserved Asn residue at the end of the β4 region. In many class I MTases, including the N-terminal domain of MnmC and the present *A. aeolicus* DUF752, this motif constitutes an essential element of the nucleotide binding pocket (27).

**FIGURE 2. Sequence alignment of DUF752, other homologous proteins, and the N-terminal domain of MnmC2 of the bifunctional MnmC.** The sequence alignment was created with ESPript (51). Conserved residues are colored red. The highly conserved residues are represented by white letters within red rectangles. The conserved motifs in the class I MTases are underlined by heavy blue lines. The signature motif DXF in motif IV is indicated by blue stars. The orange star indicates the residue proposed to interact with the substrate RNA. Residues interacting with the cofactor, AdoMet, are indicated by blue triangles. Filled triangles represent the residues interacting through side chains, and empty triangles represent the residues interacting through backbones. GI numbers are given in parentheses as follows: *A. aeolicus* VF5 (15606976); *Methanocaldococcus jannaschii* DSM 2661 (15668851); *T. thermophilus* HB8 (55980847); *Synechococcus elongatus* PCC 7942 (81300750); *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 (218562880); *Chromobacterium violaceum* ATCC 12472 (34497397); *E. coli* str. K-12 substr. W3110 (85675355).

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**Overall Structure of DUF752**—The overall tertiary structure of monomeric *A. aeolicus* DUF752, determined at 2.5 Å resolution, shows a central core domain with the canonical secondary structure characteristic of the class I MTases, with additional...
N- and C-terminal modules (Fig. 3, compare A and B). The additional N-terminal module (in yellow in Fig. 3) consists of two \( \alpha \)-helices and three \( \beta \)-strands, whereas the C-terminal module (in red in Fig. 3) consists of two \( \alpha \)-helices. The \( \alpha \)-helices of both termini are close together (Fig. 3, A and B). The \( \beta \)-sheet (\( \beta_1 \) to \( \beta_3 \)) with \( \alpha_1 \) and \( \alpha_2 \) in the N-terminal module surround the cofactor, AdoMet, along with the MTase fold (see below). In fact, this \( \beta \)-sheet covers the AdoMet binding pocket and limits cofactor access to the solvent (see arrow in Fig. 3B). This feature is characteristic of DUF752 and all of the MnmC2 family proteins we examined (Fig. 2). In contrast, in other tRNA methyltransferases, the AdoMet is usually exposed to the solvent. The C-terminal extra sequence (\( \alpha_11 \) and \( \alpha_12 \)) of DUF752 may form part of the tRNA binding pocket, in which charged residues and aromatic amino acids could play important roles in tRNA recognition and/or binding.

A DALI search confirmed that the MTase domain (blue background in Fig. 3A) shares high similarity with the N-terminal (MnmC2) domain of the bifunctional E. coli MnmC we reported previously (supplemental Table S1) (27). However, a few other MTases also show significant structural similarities, including guanidinoacetate \( N \)-methyltransferase interacting with a small cellular metabolite and various MTases interacting with RNA, such as TrmI (tRNA-\( m^1A58 \) \( N \)-methyltransferase), mRNA cap guanine-N7 methyltransferase, RsmC (16S rRNA-\( m^2G1207 \) \( N \)-methyltransferase), and Trm1 (tRNA-\( m^2G \) \( N \)-methyltransferase (supplemental Table S1).

Within this methyltransferase domain, the residues constituting the AdoMet binding pocket are well conserved between DUF752 and the MnmC2 domain of the bifunctional MnmC. As shown in Fig. 3C, a network of amino acid interactions facilitates the recognition of the cofactor. The amino acids that are close by and/or interacting with a given atom of AdoMet are listed in supplemental Table S2, and most of them are indicated by triangles in Fig. 2. Notably, the carboxyl group in the methionine moiety is recognized by the enzyme backbone from residues Leu-107 to Asn-110, which compose part of the conserved (GX)GAGXN in motif I (Fig. 2). The N1 position of the adenine moiety is recognized by a hydrogen bond with the backbone of Ala-175 in motif III, whereas the amino group (N6)
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in the adenine is recognized by the side chains of the conserved Asp-174 in motif III and Glu-202 in motif IV. Furthermore, the N7 position of the adenine ring hydrogen bonds with the side chain of Asn-200, adjacent to the conserved DXFXP sequence in motif IV. The 2'- and 3'-hydroxyl groups of the ribose moiety of AdoMet are almost nonexistent. The methyl donor and bulk B. subtilis tRNA as the substrate. Because B. subtilis lacks a gene encoding either MnmC or a “stand-alone” MnmC1 (FAD oxidase domain), but possesses genes encoding the MnmE, MnmG, MnmA, and TrmL proteins (35), the DUF752. Under these experimental conditions, the MTase activity of DUF752, although the full activity of the FAD oxidase domain MnmC1, in the bifunctional MnmC.

MTase Activity of DUF752—To further investigate whether A. aeolicus DUF752 functions in the same manner as the E. coli MnmC2 enzyme, the MTase activity was monitored in vitro, using the purified recombinant enzyme, radiolabeled AdoMet as the methyl donor and bulk B. subtilis tRNA as the substrate. Because B. subtilis lacks a gene encoding either MnmC or a “stand-alone” MnmC1 (FAD oxidase domain), but possesses genes encoding the MnmE, MnmG, MnmA, and TrmL proteins (35), the DUF752 was fused to its oxidase domain MnmC1, in the bifunctional MnmC.

In summary, the overall structure of A. aeolicus DUF752, in complex with its cofactor AdoMet, is very similar to that of the MnmC2 domain of E. coli MnmC, as we determined previously (27). All of the observations mentioned above support the idea that the monofunctional methyltransferase DUF752 and of the partially conserved Glu-133 in motif II. The location of the carboxyl group of the conserved Asp-193 probably allows the formation of a hydrogen bond with the amino group in the AdoMet. Finally, the base moiety of AdoMet is recognized by the partially conserved Glu-133 in motif II. The location of the carboxyl group of the conserved Asp-193 probably allows the formation of a hydrogen bond with the amino group in the AdoMet.

FIGURE 4. Measurements of MTase activities in vitro. A quantitative diagram of the time courses of the MTase assays. The reactions were performed with unlabeled B. subtilis total tRNA and [14C]AdoMet. Experimental details are provided under “Experimental Procedures” and the text.

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mnm\textsuperscript{5}s\textsuperscript{2}U but no nm\textsuperscript{5}s\textsuperscript{2}U. In wild-type Salmonella enterica grown logarithmically in rich medium, only a small amount of cmm\textsuperscript{5}s\textsuperscript{2}U was observed, originating from tRNA\textsuperscript{Glu} (36). Thus, wild-type E. coli has the capacity to synthesize cmm\textsuperscript{5}s\textsuperscript{2}U, and its subsequent enzymatic transformation is sensitive to the physiological conditions and the substrate tRNA. Because the nm\textsuperscript{5}s\textsuperscript{2}U intermediate is rarely observed, one can conclude that under in vivo conditions it is quickly converted to the mature mnm\textsuperscript{5}s\textsuperscript{2}U, a situation that is consistent with the in vitro methylation reaction occurring more quickly than the cleavage reaction (22, 24). In contrast, in the hydrolysate derived from tRNA isolated from the ΔmnmC::Amp E. coli strain, only the intermediate product cmm\textsuperscript{5}s\textsuperscript{2}U was evident, whereas no nm\textsuperscript{5}s\textsuperscript{2}U was detected (Fig. 5B). Thus, by abolishing the activity of the MnmC enzyme, the cmm\textsuperscript{5}s\textsuperscript{2}U intermediate accumulates in E. coli, a situation one would expect in any organism lacking the bifunctional mnmC gene, if the glycine pathway was operating (Fig. 1A, upper pathway).

An interesting result is shown in Fig. 5C, where despite the absence of the bifunctional mnmC gene in the A. aeolicus genome the only s\textsuperscript{2}U derivative present, besides the expected m\textsuperscript{5}s\textsuperscript{2}U, was cmm\textsuperscript{5}s\textsuperscript{2}U, and notably, no cmm\textsuperscript{5}s\textsuperscript{2}U was detected. This result allowed us to conclude that, in contrast to the situation in E. coli (12), in A. aeolicus the intermediate nm\textsuperscript{5}s\textsuperscript{2}U34 is the major product catalyzed by the enzymes MnmE, MnmG, and MnmA, and in turn it becomes fully methylated to the final derivative mnm\textsuperscript{5}s\textsuperscript{2}U via the ammonium pathway depicted in Fig. 1A (lower part). Because no traces of cmm\textsuperscript{5}s\textsuperscript{2}U could be detected in the tRNA originating from A. aeolicus (Fig. 5C), the production of this intermediate by the alternative glycine pathway is obviously insignificant (Fig. 1A, upper part).

The large peak eluting after s\textsuperscript{2}U in Fig. 5C corresponds to m\textsuperscript{5}s\textsuperscript{2}U (s\textsuperscript{2}T), which was demonstrated to exist at position 54 in the T loop of tRNA isoacceptors in thermophilic organisms, such as Thermus thermophilus (37) and Pyrococccus furiosus (38), and obviously also in A. aeolicus (this work). As a matter of fact, the A. aeolicus genome contains the trnFO and ttuA genes, which encode a tRNA-m\textsuperscript{5}sU54 methyltransferase (25) and a tRNA U54 thiolase (39), respectively.

DISCUSSION

In A. aeolicus, the Major Route for Enzymatic Formation of mnm\textsuperscript{5}s\textsuperscript{2}U34 Depends on Use of Ammonium Rather Than Glycine—We showed that the A. aeolicus DUF752 protein is a homolog of the MnmC2 domain of the bifunctional E. coli MnmC and shares the same cellular function. Not only are the sequences and structures very similar but also the purified recombinant DUF752 catalyzes the same methylation reaction in vitro as the MnmC2 domain of the E. coli bifunctional MnmC. Despite the absence of the FAD-dependent oxidase MnmC1 homolog, the A. aeolicus tRNA contains the same mnm\textsuperscript{5}s\textsuperscript{2}U34 modification as in tRNAs from other bacteria encoding the bifunctional mnmC gene. These observations strongly support the recent results showing that in E. coli two alternative pathways exist for the biosynthesis of the mnm\textsuperscript{5}s\textsuperscript{2}U34 derivative. One mainly uses glycine as a cofactor and requires the combined activities of the FAD-dependent MnmC1 plus the methyltransferase MnmC2, and a second minor one, identified only under special experimental conditions, uses ammonium as a cofactor and requires the MnmC2 methyltransferase activity (Fig. 1A) (12, 17).

Given the complete absence of cmm\textsuperscript{5}s\textsuperscript{2}U34 in bulk A. aeolicus tRNA (Fig. 5C), we concluded that in A. aeolicus, and probably in other bacteria lacking the gene encoding an MnmC1 homolog, the shortcut ammonium-dependent metabolic pathway is prevalent. In addition, because the tRNA harboring the nm\textsuperscript{5}s\textsuperscript{2}U modification does not accumulate in either wild-type E. coli or A. aeolicus (Fig. 5), under the usual laboratory experimental conditions for cell growth, the nm\textsuperscript{5}s\textsuperscript{2}U34 derivative either does not accumulate or is rapidly methylated by the MnmC2/DUF752 enzyme. The same situation existed when recombinant E. coli MnmC or a mixture of the MnmC1 and MnmC2 domains of E. coli were tested under in vitro conditions (22, 24). In addition, consistent with the finding that the nm\textsuperscript{5}s\textsuperscript{2}U intermediate is rarely observed in tRNA (22), the in vitro methylation reaction was shown to be much faster than the cleavage reaction (24).

The M. capricolum and B. subtilis genomes lack the genes encoding the bifunctional MnmC protein or a monofunctional MnmC2. Instead, they encode the MnmE, MnmG, MnmA, and MnmC2. Except that taurine, instead of glycine, is used by the GTPBP3/MTO1 enzymes, the homologs of bacterial MnmE-MnmG, to catalyze m\textsuperscript{5}s\textsuperscript{2}U34 and m\textsuperscript{5}s\textsuperscript{2}U34 formation in a subset of their tRNAs (6, 41). Evidently, the type of biosynthetic pathway leading to mnm\textsuperscript{5}s\textsuperscript{2}U34 strongly support the recent results showing that in E. coli two alternative pathways exist for the biosynthesis of the mnm\textsuperscript{5}s\textsuperscript{2}U34 derivative. One mainly uses glycine as a cofactor and requires the combined activities of the FAD-dependent MnmC1 plus the methyltransferase MnmC2, and a second minor one, identified only under special experimental conditions, uses ammonium as a cofactor and requires the MnmC2 methyltransferase activity (Fig. 1A) (12, 17).

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The M. capricolum and B. subtilis genomes lack the genes encoding the bifunctional MnmC protein or a monofunctional MnmC2. Instead, they encode the MnmE, MnmG, MnmA, and TrmL enzymes (35, 40). In these bacteria, the C5 atom of the wobble U34 of the tRNAs specific for Gln, Lys, Glu, Arg, Gly, and Leu were shown to harbor only cmm\textsuperscript{5}s\textsuperscript{2}U, cmm\textsuperscript{5}s\textsuperscript{2}U34, or cmm\textsuperscript{5}s\textsuperscript{2}U34 derivatives (4, 5). The glycine-dependent metabolic pathway (Fig. 1A, upper part) is obviously the dominant route used by these bacteria. The situation in mammalian mitochondria is similar, except that taurine, instead of glycine, is used by the GTPBP3/MTO1 enzymes, the homologs of bacterial MnmE-MnmG, to catalyze m\textsuperscript{5}s\textsuperscript{2}U34 and m\textsuperscript{5}s\textsuperscript{2}U34 formation in a subset of their tRNAs (6, 41). Evidently, the type of biosynthetic pathway leading to Xm\textsuperscript{5}s\textsuperscript{2}U34 derivatives in a subset of tRNAs strongly depends on the organism and the organelles considered and probably the physiological/environmental growth conditions of the cells as well.

Conserved Aspartic Acid Residue in the DUF752/MnmC2 Enzyme Plays an Essential Role in the Methylation Reaction, as
in the Other Class 1 N-Methyltransferases—As mentioned above, the structure of A. aeolicus DUF752 is highly homologous to the N-terminal MnmC2 domain of the bifunctional E. coli MnmC-fused protein (Fig. 6A). The electrostatic potentials of DUF752 (Fig. 3B, right panel) and E. coli MnmC (27) indicated that tRNA binds to both proteins on the same side. Furthermore, they both possess the conserved DXF sequence in motif IV (Asp-193 and Phe-195 in A. aeolicus and Asp-178 and Phe-180 in E. coli, marked with blue stars in Fig. 2). This aspartic acid residue in the DXF sequence is highly conserved in all of the DUF752/MnmC2 sequences examined so far (Fig. 2) (21, 27). In the crystal structures of DUF752 and the MnmC2 domain, this motif surrounds

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the methyl group donor of AdoMet (Fig. 3C). The corresponding region of many class I MTases involved in N-methylation reactions has a semi-conserved (D/N/S)PPY motif (34). In these MTases, the first (D/N/S) and the second proline residues form hydrogen bonds with the cofactor, with the nitrogen nucleophiles oriented toward the methyl group of AdoMet (34, 42, 43). Interestingly, the Trm1 protein, which also belongs to the class I N-methyltransferase family and catalyzes the dimethylation of the exocyclic amino group of the G26 residue in tRNAs (44–48), possesses a DPF motif (49). Based on the structure of Pyrococcus horikoshii Trm1, the first aspartic acid residue in the DPF motif was proposed to function as a nucleophile, by attracting one proton of the amino group of the target G26 (49). The superimposition of DUF752 on Trm1 indicated that the conformations of the AdoMet binding pockets of both proteins are similar (Fig. 6B), suggesting that the Asp-193 residue in the DAF motif of A. aeolicus DUF752 plays the same role as the corresponding residue in the DPF motif (Asp-138) of P. horikoshii Trm1. In this scenario, the carboxyl group of Asp-193 in DUF752 is a catalytic base that attracts the proton of the amino group of the nm5U34-containing tRNA. In synergy with other active site amino acids, this Asp-193 residue could then catalyze the nucleophilic attack on the methyl moiety of AdoMet (Fig. 6C). Notably, the structure and the surface potential of A. aeolicus DUF752 are arranged in such a way that the nm5U34-target base of tRNA enters the catalytic pocket from the opposite side of the proposed tRNA-binding sites in the P. horikoshii and A. aeolicus Trm1 enzymes (49, 50). In the Trm1 enzymes, the exocyclic amine group of the G26 target nucleoside of the tRNA is stabilized through a stacking interaction with a phenylalanine residue (Phe-140 in P. horikoshii and Phe-134 in A. aeolicus) in motif X. However, in the MnmC2/DUF752 family, it is more likely that the substrate is stabilized by a conserved Tyr (Tyr-225 in A. aeolicus) in motif VI (Fig. 6C), whereas a phenylalanine is prevalent at the corresponding position in MnmC2 of the bifunctional E. coli MnmC (Phe-210 in E. coli) (27). In the MnmC2/DUF752 family, the additional N- and C-terminal modules prevent the substrate tRNA from entering the catalytic pocket from the same side, as in the Trm1 enzymes. Instead, the MnmC2/DUF752 enzyme forms a possible tRNA-binding site on the opposite side, composed of the conserved positive residues (Fig. 3B, right panel). This narrow binding pocket leading to the AdoMet cofactor is considered to accommodate the longer 5-aminomethyl aduct of the substrate.

Taken together, the monofunctional DUF752 was proved to possess the same functional and structural properties as the MnmC2 domains of the bifunctional MnmC family of enzymes. Thus, we propose the designation of all of these bacterial enzymes with the same acronym, MnmC2.

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