Identification of a novel gene encoding a flavin-dependent tRNA: m^5U methyltransferase in bacteria—evolutionary implications

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ABSTRACT

Formation of 5-methyluridine (ribothymidine) at position 54 of the T-psi loop of tRNA is catalyzed by site-specific RNA methyltransferases (tRNA:m^5U-54 MTase). In all Eukarya and many Gram-negative Bacteria, the methyl donor for this reaction is S-adenosyl-L-methionine (S-AdoMet), while in several Gram-positive Bacteria, the source of carbon is N^6, N^10-methylenetetrahydrofolate (CH\(_2\)H\(_4\)folate). We have identified the gene for Bacillus subtilis tRNA:m^5U-54 MTase. The encoded recombinant protein contains tightly bound flavin and is active in Escherichia coli mutant lacking m^5U-54 in tRNAs and in vitro using T7 tRNA transcript as substrate. This gene is currently annotated gid in Genome Data Banks and it is here renamed trmFO. TrmFO (Gid) orthologs have also been identified in many other bacterial genomes and comparison of their amino acid sequences reveals that they are phylogenetically distinct from either ThyA or ThyX class of thymidylate synthases, which catalyze folate-dependent formation of deoxyribothymine monophosphate, the universal DNA precursor.

INTRODUCTION

Transfer RNAs in all living organisms contain a number of nucleosides that are post-transcriptionally modified on the base and/or the 2'-hydroxyl group of the ribose (1). One such common modified nucleoside is 5-methyluridine (m^5U, also designated T for ribothymidine). This C^5^-methylated uridine is invariably found at position 54, in the so-called T-psi loop of tRNA of almost all Bacteria and Eukarya (2). In thermophilic Bacteria, such as Thermus thermophilus, it is further hypermodified to a 2-thio-derivative [m^5S^2U] or s^2T, reviewed in (3), while in certain Eukarya, a 2'-O-methyl-derivative is occasionally found [m^5Um (2)].

Site-specific methylation of U-54 in Escherichia coli tRNA is catalyzed by tRNA:m^5U-54 methyltransferase (EC.2.1.1.35). This enzyme, initially designated RUMT for RNA uridine methyltransferase, was the first RNA modification enzyme discovered that acts at the polynucleotide level (4,5). This enzyme is also called TrmA (tRNA methyltransferase A), and a gene trmA encoding this enzyme was first identified in E.coli (6,7). From the standpoint of mechanism and specificity, the tRNA:m^5U-54 methyltransferase of E.coli is one of the best characterized RNA modification enzymes [reviewed in (8)]. In the majority of RNA methyltransferases studied so far [reviewed in (9,10)], RUMT uses S-adenosylmethionine (S-AdoMet) as the methyl donor. Automated bioinformatic approaches have included all trmA and TRM2 homologs in the same cluster of orthologous genes [COG2265, see http://www.ncbi.nlm.nih.gov/COG/, (11)]. This cluster contains a superfamily of S-AdoMet-dependent RNA: m^5U MTases that are specific not only for uridine at position 54 of tRNA, but also paralogs that function in uridine methylation in other RNAs [e.g. U-747 or U-1939 in E.coli 23S rRNA (12,13)].

Earlier studies have indicated that not all bacterial tRNA: m^5U-54 MTases use S-AdoMet as methyl donor. For example, in Enterococcus faecalis (formerly Streptococcus faecalis) and Bacillus subtilis, it was reported that the carbon donor of the methyl group is N^6, N^10-methylenetetrahydrofolate (CH\(_2\)H\(_4\)folate) [(14) and references therein]. The first indication for this came from an observation that bulk tRNAs isolated from folate-deprived E.faecalis cells lacked m^5U-54 in their T-psi loop (15). Moreover, in B.subtilis and Micrococcus lysodeikticus, trimethoprim, a specific inhibitor of bacterial dihydrofolate reductase, inhibits formation of

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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m^5U-54 in vivo (16), indicating that in these Gram-positive bacteria, the carbon source used in tRNA methylation derives from the folate pool. The results of these in vivo studies were later confirmed by demonstrating that in vitro activity of purified tRNA: m^5U-54 MTase of E. faecalis not only requires CH_2H_4folate but also reduced flavin adenine nucleotide (FADH\_2) (14,17,18), thus forming a distinct class of tRNA:m^5U-54 MTases (EC:2.1.1.74). Strikingly, this observation is reminiscent of the enzymatic mechanism that has been described for the alternative flavin-dependent ThyX class of thymidylate synthases (EC:2.1.1.148) (19–21), but differs from the reaction catalyzed by a canonical thymidylate synthase ThyA, which uses CH_2H_4folate both as a carbon source and as a reductant [EC:2.1.1.45; reviewed in (22)]. Moreover, in the case of ThyX catalysis, it has been recently demonstrated that a hydride from NAD(P)H is transferred, via a FAD cofactor to reduce the methylene group, to a methyl residue (23–26). The gene encoding the folate-dependent tRNA:m^5U-54 MTase has not yet been identified. It is, therefore, not known whether these analogous folate-dependent methylation reactions, involved in RNA or DNA metabolism, are catalyzed by distantly related enzymes, possibly originating from the early RNA World, or, on the contrary, represent independent catalytic mechanisms.

Benefiting from large-scale microbial sequencing and structural genomics projects, we predicted that bacterial Gid proteins would correspond to a novel class of bacterial site-specific tRNA:m^5U-54 MTases. This prediction was confirmed through genetic studies and biochemical analyses of tRNA molecules isolated from B. subtilis wild-type and mutants strains. In vitro characterization of the purified recombinant B. subtilis tRNA:m^5U-54 MTase indicates that this protein alone is sufficient for tRNA methylation reaction. Our studies further indicate that despite the fact that thymidylate synthase ThyX and Gid proteins catalyze a similar methylation reaction, they lack detectable sequence, and probably structural similarity. Our analyses suggest that the enzymes methylylating nucleotides in tRNA and DNA precursor using CH_2H_4folate and NAD(P)H/FAD as carbon donor and reductant, respectively, have independent evolutionary origins.

### MATERIALS AND METHODS

#### Strains

B. subtilis strain BFS2838 carrying inactivated gidQermR gene was kindly provided by S. Seror [European functional analysis project of B. subtilis (http://bacillus.genome.jp/bisorfbin/BSORF_data_view.pl?Accession=BG11008)]. E. coli strain GRB113 (meta, trmA5, zj-90::Tn10), encoding an inactive TrmA protein was a kind gift from G. R. Björk, Umeå University, Sweden. E. coli Sure\_R strain [e14\^- (McrA\^-) ΔmcrCB-hsdSMR-mrr]171 endA1 supE44 thi-1 gyrA96 relA1 lac recB reC sbcC umuC::Tn5 (Kan\^-) urvC [F\^ proAB lacPZΔM15 Tn10 (Tet\^-)] was purchased from Stratagene.

#### Construction of an N-terminal His\_6-tagged BsuGidA and BsuGid-overexpressing plasmids and purification of the corresponding recombinant proteins

The gidA (GIDA_BACSUB; P25812) and gid genes (GID_BACSU; P39815; renamed trmA\_FO in this work) were amplified by PCR from B. subtilis strain 168 DNA, using Pfu DNA polymerase (Promega) and the following primers (sequence in small characters correspond to genome sequence): gidAfw (CAGGATCCatggggtaagccagccgcaacatc) and gidArev (CCCTTGGGAaattctaatcagttttcgcgc). The resulting 1903 or 1314 bp PCR products were then digested with BamHI and SmaI, respectively, and cloned into pQE80L to generate pQE80L-BSuGidA or pQE80L-BSuGid. To purify recombinant GidA and Gid proteins, pQE80L-BSuGidA was transformed into E. coli Sure\_R strain, and pQE80L-BSuGid was transformed into Sure\_R or GRB113 (trmA5) strain. Resulting strains were grown at 37°C in 500 ml of Luria–Bertani (LB) medium (Invitrogen) containing 100 μg/ml ampicillin until OD\_600 = 0.6. After induction of Gid or GidA protein expression by isopropyl β-D-thiogalactopyranoside (IPTG) (VWR International, final concentration = 1 mM), the cultures were further grown at 37°C for 3 h. After harvesting the cells by centrifugation, the pellet was flash-frozen in liquid N\_2 and stored at −80°C. Frozen cells were thawed on ice and resuspended in 5 ml of lysis buffer (50 mM sodium phosphate, pH 7.6, 300 mM NaCl, 10% glycerol and 20 mM imidazole) containing 5 μl Protein Inhibitor Cocktail (PIC, Sigma) and 1.5 μl β-mercaptoethanol. Cells were broken by 2 freeze (liquid N\_2)/thaw (37°C) cycles and ultrasonication. The lysate was centrifuged for 15 min at 10 000 g at 4°C. Supernatant was loaded onto 2 ml of Ni-NTA resin and washed with 25 ml of lysis buffer. Gid or GidA proteins were eluted with 10 ml elution buffer (same as lysis buffer, but containing 250 mM imidazole). Yellow fractions, containing the Gid or GidA protein, were pooled (to ∼3 ml of total volume) and dialyzed against 500 ml of 30 mM HEPES buffer, pH 7.5, containing 200 mM NaCl and 10% glycerol. Protein was aliquoted, flash-frozen in N\_2 and stored at −80°C. To measure any cofactor release from Gid, 5 μg of protein was diluted in 100 μl of distilled water and incubated for 5 min at 90°C. The sample was centrifuged at 10 000 g for 15 min. Absorption and fluorescence spectra of the obtained supernatant were measured.

#### Preparation of cell-free extracts

Cell-free extracts of B. subtilis strains 168 (wild-type) and BFS2838 (gidQermR) were prepared from an exponentially growing cell culture at 37°C. After centrifugation and washing the cell pellet with lysis buffer (25 mM Tris–HCl buffer, pH 7.5, 10 mM MgCl\_2, 25 mM KCl and 2 mM DTT), it was resuspended in a 1.5 vol of lysis buffer containing 1% (v/v) of PIC, Sigma. An S10 cell-free extract was obtained after ultrasonication and centrifugation for 15 min at 10 000 g. Further centrifugation of supernatant for 1 h at 4°C resulted in S100 cell-free extracts. Cell-free extracts of E. coli strains pQE80L-BsuGid/GRB113 (trmA5) and pQE80L/GRB113 (trmA5) were prepared similarly as for the protein purification, except that they were grown at 37°C to an OD\_500 of 0.8 in 10 ml of liquid Luria Broth with 100 μg/ml carbenicillin. After the Gid protein induction, harvesting of cells, resuspension in 500 μl lysis buffer, cell disruption by ultrasonication and centrifugation, the S10 cell-free extract was produced.
Enzymatic activity assays

([α-32P]UTP-labeled yeast tRNAAsp transcript, used for determining the tRNA:m5U-54 MTase activity of TrmFO (Gid) protein, was prepared and purified as described elsewhere (27,28). A total of 50–100 fmol of [32P]-labeled tRNAAsp were incubated at 37°C in a 50 μl reaction mixture containing 40 mM N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]-Na buffer (HEPES- Na, Sigma) at pH 7.0, 0.25 mM FAD, Fluka, 0.5 mM NADH (reduced nicotinamide adenine dinucleotide, Sigma), 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.25 mM (6R)-N5,N10, CH2H2PteGlu-Na2 (methylene tetrahydrofolate, provided by Dr R. Moser, Merck-Eprova, AG, Switzerland), 5 mM DTT (Promega), 15 U of RNase inhibitor (Fermentas) and 10–25 μg of total protein of a B.subtilis or E.coli cell-free extract. At the end of the incubation period, modified tRNA was extracted and digested with nuclease P1 (Roche), the modified nucleoside was recovered, digested by nuclease P1, and the resulting radiolabeled nucleotides were analyzed by 2D-TLC as described previously (29). Methylating activity of purified recombinant BsuGidA protein (1 mg per test) and BsuGidA protein (1 mg per test) were tested using the same experimental conditions as above. Activity of the MnmC enzyme on bulk tRNAs from B.subtilis strains 168 or BFS2838 (gidA5) was tested as follows: five microgram of purified recombinant MnmC protein (provided by Dr L. Droogmans, University of Brussels, Belgium) was added to 300 μl of a reaction mixture containing 50 mM Tris–HCl, pH 8.0, 20 mM NH4Cl, 62 μM [methyl-14C]-AdoMet (53 Ci/mmol, Amersham) and 100 μg of bulk B.subtilis tRNAs. After 1 h incubation at 37°C, tRNA was recovered, digested by nuclease P1, and the resulting radiolabeled nucleotides were analyzed by 2D-TLC as described previously (30).

Isolation of tRNA and chromatographic analysis of tRNA hydrolysates

Bulk tRNAs of B.subtilis strains 168 and BFS2838 (gidA5) were obtained essentially as described previously (31), except that the tRNA deacylation step was omitted and a monoQ column (5 ml from Biorad) was used instead of DEAE-cellulose. For bulk tRNAs from E.coli GR113 (trmA5), transformed by pQE80L-basGid or pQE80L (control experiment), cell cultures were first grown at 37°C in 200 ml of liquid Luria Broth in the presence of 100 μl/ml carbenicillin. At OD600 = 0.6, IPTG was added to the final concentration of 1 mM, and the cells were grown for additional 3 h at 37°C before to be collected in the cold by centrifugation and purified as above. Obtained purified bulk tRNAs were completely degraded to nucleosides with P1 nuclease and alkaline phosphatase (Sigma) and the resulting hydrolysates analyzed by high performance liquid chromatography (HPLC) on a Supelcosil LC18 column (Supelco) with Waters HPLC instrument, as described previously (32).

RESULTS

Comparative genomics identifies a candidate gene encoding a new family of flavin-dependent methyltransferases

An enzyme of two identical subunits of ~58 kDa that catalyzes the site-specific formation of 5-methyluridine in position 54 (m5U-54) of tRNA using CH2H2folate as a source of one-carbon unit and a combination of coenzymes NAD(P)H/ FAD as reductant, has been purified from E.faecalis (33). We attempted an identification of the gene encoding this activity (described under EC 2.1.1.74) based on the facts that a folate-dependent pathway for tRNA methylation exists in some Gram-positive Bacteria species [except Geobacillus stearothermophilus (34,35)], whereas an S-AdoMet-dependent enzyme is used instead in Eukarya [e.g. Saccharomyces cerevisiae (36)], in gamma-proteobacteria [e.g. E.coli (7)] and in a few Archaea [e.g. Pyrococcus furiosus (37)].

Our primary searches used an updated version of COG database (http://www.ncbi.nlm.nih.gov/COG/), which currently consists of 4873 gene families (11). Using the phylogenetic distribution analysis tool of this database, we obtained a list of 155 COGs (~3% of total number of families) that are present in B.subtilis and Bacillus halodurans, but absent in Archaea, Eukarya and gamma-proteobacteria (data not shown). We have no data for E.faecalis, M.lysoideitcus and G.stearothermophilus, as they are not included in the current data release. Next, among the 155 candidates, we searched for the presence of a characteristic ‘GXGXXG’ motif that is part of the conserved Rossman-fold found in a large number of FAD binding proteins [reviewed in (38)]. As a result, one COG family (COG1206) emerged as an evident protein family encoding a putative tRNA:m5U-54 MTase. These COG1206 proteins, also designated as Gid proteins (in reference to the B.subtilis protein) are: (i) currently annotated as ‘NADPH(FAD)-utilizing enzymes possibly involved in translation’, (ii) contain a readily identifiable FAD binding motif ‘GXGXXG’ (in fact G-X-G-A) and (iii) their molecular weight is ~50 kDa, in close agreement with the 58 kDa determined on SDS–PAGE gels for the α-subunit of the E.faecalis folate-dependent tRNA:m5U-54 MTase (33).

COG1206 proteins have a wide phylogenetic distribution

Systematic screening of ~200 fully sequenced genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), using pattern-hit initiated BLAST algorithm (39) and B.subtilis Gid (GID_BACSU; P39815) as a query, identified ~80 bacterial species containing a gid gene, whereas no hits were found in archaean nor eukaryal genomes. This phylogenetic distribution of gid genes (Figure 1) is much wider than initially anticipated. In addition to the expected Gram-positive bacteria (Bacillales and Lactobacillales), a gene for Gid-like protein is also found in alpha-proteobacteria, delta-proteobacteria and cyanobacteria. Phylogenetic analyses of a subset of Gid orthologs, using neighbor-joining trees performed with ClustalX program (40), indicate that their phylogeny is congruent with species phylogeny, suggesting a relatively ancient bacterial origin for Gid proteins (Figure 1).

The Gid protein of B.subtilis is involved in m5U-54 formation in tRNA

To determine whether Gid proteins are involved in the biosynthesis of m5U-54, the presence of this methylated nucleoside was analyzed in B.subtilis tRNA isolated from a BFS2838 (gidA5) strain, lacking functional Gid protein (kindly provided by S. Seror, University of Paris XI).
No obvious phenotype has been described for this \textit{B.subtilis} strain (see \url{http://locus.jouy.inra.fr/cgi-bin/dev/chiapell/strain_pheno_old.pl?STRAIN=BFS2838}). Bulk tRNAs from the mutant strain and the corresponding wild-type strain \textit{B.subtilis} 168 were extracted, and their nucleoside contents were analyzed by HPLC as described in Materials and Methods. Results in Figure 2A and B clearly demonstrate that m$^5$U is absent in the tRNA from the \textit{gid} \textit{W}ermR mutant, whereas tRNA of the wild-type strain 168 contains the m$^5$U modification. The small residual peak eluting at the same position as m$^5$U in Figure 2B was identified as inosine through its characteristic UV absorbance spectrum. The maximum wavelength for inosine is at 250 nm (Figure 2D), compared with 267 nm for 5-methyluridine (Figure 2C).

The absence of C$^5$-methylation activity for U-54 in \textit{gid}\textit{W}ermR mutant strain was further confirmed by testing the corresponding methylation activity in cell extracts. Thus, [UTP-$^{32}$P]-radiolabeled T7-transcript of a synthetic yeast tRNA\textsuperscript{Asp} gene was used as substrate, and incubations were performed in the presence of CH$_3$H$_2$folate, NADH/NADPH and FAD as indicated in Materials and Methods. After incubation, the tRNA was digested into 5'-monophosphate nucleosides and the hydrolysate was analyzed by 2D-TLC. The radiolabeled spots, corresponding to [$^{32}$P]-labeled UMP-derivatives were detected by autoradiography. As shown in Figure 2A and B (insets), while the wild-type cell extract was able to catalyze the formation of m$^5$U-54 \textit{in vitro}, the extract from the \textit{gid}\textit{W}ermR mutant strain did not catalyze such a methylation reaction, thus indicating that the \textit{gid} gene product is involved in the production of m$^5$U-54 in tRNA.

\textbf{The Gid protein of \textit{B.subtilis} is sufficient for methylation of U-54 in \textit{E.coli} tRNA in vivo}

To investigate whether the \textit{B.subtilis} Gid protein alone can substitute for the S-AdoMet-dependent \textit{E.coli} TrmA protein for the formation of m$^5$U-54 \textit{in vivo}, we cloned the \textit{B.subtilis} gid into an \textit{E.coli} expression vector pQE80L, under the control of an IPTG inducible promoter. The resulting plasmid, pQE80L-BsuGid was transformed into an \textit{E.coli} strain.
GRB113, carrying trmA5 mutation. This E.coli strain grows normally in LB medium but completely lacks S-AdoMet-dependent tRNA:m^5U-54 MTase activity (41). After gid expression for 3 h, the cells were collected by centrifugation and divided into two parts. Bulk tRNA was purified from one part, while the remaining cell pellet was used to prepare an S10 cell extract (see Materials and Methods). HPLC analysis of P1/alkaline phosphatase-treated bulk tRNA hydrolysate demonstrated the presence of m^5U nucleoside in the E.coli trmA5 strain transformed by pQE80L_{Bsu}Gid (Figure 3A), while in the control E.coli mutant strain, transformed by unmodified expression vector, no m^5U was detectable.
(Figure 3B). As described above with *B. subtilis* bulk tRNAs (Figure 2), we verified that the very small peak migrating at the position expected for m^5^U in the HPLC analysis of tRNA hydrolysate of the control strain corresponds to inosine (see UV-spectrum in Figure 3D, compare with Figure 3C for m^5^U). In parallel, the S10 cell extract was incubated together with appropriate cofactors and [UTP-^32^P] labeled yeast tRNA^A^p transcript. The P1-hydrolyzate of the resulting modified tRNA was then analyzed by 2D-TLC. Results in Figure 3A and B (insets) indicate that m^5^U-54 in tRNA^A^p is formed only when a cell-free extracts from the *E. coli trmA5* strain transformed with pQ80L-BsuGid, confirming that the *B. subtilis* Gid protein efficiently modified tRNAs under the physiological conditions of *E. coli* cells.

**Purified recombinant bsaGid protein catalyzes the in vitro formation of m^5^U-54 in tRNA**

The *B. subtilis* Gid protein was tagged with six histidine residues at the N-terminus, and purified to near homogeneity through affinity chromatography, either from *E. coli* Sure® strain (Figure 4A) or from *E. coli trmA5* strain, both transformed with pQ80L-bsuGid. The purified protein is yellow and elutes from an S-200 gel filtration column at ~85 kDa (data not shown), suggesting that the functional form of the enzyme may be a homodimer. Heating of the protein at 90°C releases yellow cofactor that has absorption (data not shown) and fluorescence spectra (Figure 4B) characteristic for oxidized flavins. This cofactor is likely FAD that was present in 0.8 mol per 1 mol of Gid from *Myxococcus xanthus* [(42), see also below]. Qualitative experiments indicated that the purified protein catalyzes the site-specific formation of m^5^U-54 in [^32^P]-labeled yeast tRNA^A^p transcript (Figure 4C). Specific activity of the purified recombinant protein is low; nevertheless, this data reinforce observations obtained above by means of genetics. Some activity was observed without the addition of a carbon donor in the reaction mixture, suggesting that either a small amount of a carbon donor co-purifies with the enzyme or, alternatively, the purified enzyme contain tightly bound methylene or methyl intermediates. It is of note that the enzymatic test described here is highly sensitive, detecting even femtomolar amount of methylated uridine in the [^32^P]-radiolabeled substrate and has not been systematically optimized during this work.

Taking together all the above information, we now propose to rename the Gid protein as TrmFO (FO for the folate) and the corresponding gene *trmFO*, in order to differentiate them from the conventional S-AdoMet-dependent TrmA enzyme and *trmA* gene.

**Figure 4.** (A) Electrophoretic analysis of purified recombinant bsaGid protein. An SDS–PAGE analysis was performed using 11% gels, stained with Coomassie blue. Lane 1, soluble proteins from sonicated total cell-free extract of *E. coli* pQE80L-bsuGid/SURE; lane 2, S10 fraction from *E. coli* pQE80L-bsuGid/SURE; lane 3, molecular weight markers; lane 4, proteins eluted from the immobilized metal ion adsorption chromatographic column. (B) Fluorescence spectrum of a cofactor released by heat denaturation from purified bsaGid protein. The observed emission maximum (after excitation at 450 nm) at 520 nm is typical for flavin nucleotides. (C) Time course of m^5^U-54 formation catalyzed by bsaGid. The molar ratio of m^5^U over total U in yeast tRNA^A^p was evaluated over time at 37°C in the presence (closed circles) or absence (open circles) of CH_2H_4 folate.
**DISCUSSION**

The RNA methyltransferases (MTases) add methyl groups to the base or the ribose 2'-hydroxyl of ribonucleotides during the complex process of RNA maturation. The great majority of these MTases use S-AdoMet as methyl donor [reviewed in (10)]. However, at least in the case of \( m^\text{-5} \)-U-54 formation in tRNA of certain organisms, \( N^\text{5}N^\text{10} \)-methylentetrahydrofolate, together with associated oxydo-reduction coenzyme FADH\(_2\), has been shown to serve the same purpose [(14) and references therein]. This activity was first detected and the corresponding enzymes purified from *S. faecalis* almost three decades ago, but the gene encoding this folate-dependent activity had still not been identified. Here, we predicted, using prior experimental knowledge and phylogenetic distribution analyses, that Gid proteins, previously of unknown function (42,43), could correspond to such a folate-dependent tRNA methyltransferase. We have experimentally confirmed

TrmFO (Gid) and GidA proteins are two evolutionarily related families of proteins with distinct functions

TrmFO proteins of \( \approx \)50 kDa (designated Gid in Genome Data Banks) show \( \sim 40\% \) sequence similarity with another protein family referred to as GidA proteins (in reference to *E. coli* protein of \( \approx 70 \) kDa) (Figure 5). The readily detectable sequence homology, together with the currently used name ‘small GidA’ for Gid proteins [see for examples (42,43)], has created confusion regarding the putative cellular functions of TrmFO. Our studies (see also below) have now revealed that in reality, paralogous TrmFO and GidA proteins are two distinct families of proteins that probably evolved from a common ancestor but acquired different, non-overlapping cellular functions during evolution.

First, GidA proteins belong to a different cluster of orthologous genes (COG0445) and, in contrast to TrmFO proteins (belonging to COG1206), they are present in mitochondria of Eukarya and in Bacteria, with the exception of high GC% Gram-positive bacteria, such as *E. coli*. Moreover, GidA homologous genes (COG0445) and, in contrast to TrmFO proteins, are present in mitochondria of *E. coli*. Our studies (see also below) have now revealed that in reality, paralogous TrmFO and GidA proteins are two distinct families of proteins that probably evolved from a common ancestor but acquired different, non-overlapping cellular functions during evolution.

Second, consensus motifs implicated in flavin binding are slightly different in the 64 TrmFO and 203 GidA sequences analyzed (for details, see Figure 5). Moreover, GidA proteins always have an extension (or extensive insertions) that includes an additional characteristic sequence motif [P-Y-R-X(2)-T-X-R-X-E-X-R] at their C-termini.

Third, we demonstrated that *B. subtilis* TrmFO clearly methylates uridine-54 in the T-psi loop of tRNAs (see above), while *E. coli* GidA and *S. cerevisiae* MTO1 (a mitochondrial homolog of bacterial GidA) were shown to be involved in a completely different reaction, namely the multistep formation of the hypermodified uridines [5-carboxyethylaminomethyluridine (cmnm\(^5\)U) and 5-methylaminomethyluridine (cmnm\(^7\)U)] at position 34 of anticodon of a few selected tRNAs (44–48). Moreover, in agreement with the fact that the enzymatic activity of TrmFO and GidA is not overlapping, we found that the absence of U-54 methylating activity in the T-psi loop of tRNA of *B. subtilis* mutant strain BFS2838 (gid\(^\text{erm}\)) does not affect the level of conversion of U-34 into cmnm\(^5\)s\(^2\)U-34 in the anticodon loop of *B. subtilis* tRNAs. This was demonstrated by testing the capability of cmnm\(^5\)s\(^2\)U-34 residues, present in naturally occurring tRNAs of both the *B. subtilis* wild-type strain 168 and the mutant strain BFS2838 (gid\(^\text{erm}\)), to become fully modified *in vitro* into \( [1^4\text{C}]\text{mmnms}^5\text{s}^2\text{U}-34 \) upon incubation with purified recombinant *E. coli* MnmC protein. This protein is a bifunctional enzyme that is absent from *B. subtilis*. MnmC removes carboxymethyl group of cmnm\(^5\)s\(^2\)U-34 to produce cmnm\(^5\)s\(^2\)U-34 and further methylates it into cmnm\(^5\)s\(^2\)U-34 (as in naturally occurring *E. coli* tRNAs) using S-AdoMet as a methyl donor (30). The autoradiographs in Figure 6 show that the formation of cmnm\(^5\)s\(^2\)U-34 occurs equally well in the tRNAs of both the wild-type *B. subtilis* strain and the gid\(^\text{erm}\) mutant, thus clearly indicating that absence of TrmFO activity does not interfere with the GidA-dependent formation of cmnm\(^5\)s\(^2\)U-34. Conversely, we also verified that purified recombinant *B. subtilis* GidA protein does not catalyze *in vitro* a U-54 methylation reaction under the experimental conditions used for *m^5\)-U-54 formation catalyzed by CH\(_2\)H\(_4\)folate-dependent TrmFO (data not shown). Whether GidA proteins, similar to TrmFO enzymes, also act as methylases is currently unclear.

DISCUSSION

The RNA methyltransferases (MTases) add methyl groups to the base or the ribose 2'-hydroxyl of ribonucleotides during the complex process of RNA maturation. The great majority of these MTases use S-AdoMet as methyl donor [reviewed in (10)]. However, at least in the case of \( m^\text{-5} \)-U-54 formation in tRNA of certain organisms, \( N^\text{5}N^\text{10} \)-methylentetrahydrofolate, together with associated oxydo-reduction coenzyme FADH\(_2\), has been shown to serve the same purpose [(14) and references therein]. This activity was first detected and the corresponding enzymes purified from *S. faecalis* almost three decades ago, but the gene encoding this folate-dependent activity had still not been identified. Here, we predicted, using prior experimental knowledge and phylogenetic distribution analyses, that Gid proteins, previously of unknown function (42,43), could correspond to such a folate-dependent tRNA methyltransferase. We have experimentally confirmed
this prediction by showing that *B. subtilis* Gid protein (here renamed TrmFO, FO for the folate) is necessary and sufficient for ribothymidine-54 formation in the T-psi loop of rRNA both in vivo and in vitro.

Based on bioinformatics analyses, one surprising aspect of this work is that the phylogenetic distribution of the folate-dependent pathway appears much wider than originally anticipated. Nevertheless, it appears to be restricted to methylation of uridine-54 in tRNA, not m^5^U in rRNA as in the case of the S-AdoMet-dependent pathway (12,13). Strikingly, the folate-dependent TrmF proteins (COG1206) and S-AdoMet-dependent TrmA/Trm2p enzymes (COG2265) acting on tRNA appear to have mutually exclusive phylogenetic distributions (Table 1). Note that the lack of a *trmA* ortholog in a given organism is difficult to ascertain as paralogous genes that participate in S-AdoMet-dependent methylation of rRNA are also present. For instance, the *trmA*/*TRM2* gene is found in enterobacteriaceae (including *E. coli* and pseudomonaceae) as well as in all Eukarya so far sequenced. In Archaea, the *trmA/TRM2* homologs are only found in the *Pyrococcus* genus (13,37). These organisms do not contain a gene coding for a *trmF* ortholog. In contrast, orthologs of *trmF* are found in most Gram-positive bacteria (firmicutes and actinobacteria) and in several other bacterial groups (e.g. alpha and delta-proteobacteria, cyanobacteria, Table 1). Strikingly, a subset of bacteria, for instance most *Mycoplasm* species, seemingly lack either *trmFO* or *trmA* genes, suggesting that the uridine at position 54 in these tRNAs may not be methylated. Indeed, in tRNAs of *M. capricolum*, *Mycoplasma mycoides* and *Mycobacterium smegmatis*, for which the primary sequences (including modified nucleotides) are known (2,49), ribothymine-54 is indeed absent, and their bulk ribothymidine-less tRNAs can be used successfully as substrates for U-54 methylation in *E. coli* extracts (50). Interestingly, *M. mycoides* has two putative *trmF* alleles whose functional role is unclear and is worth investigation. In contrast, thermophilic *G. stearothermophilus* displays an S-AdoMet-dependent activity for m^5^U modification in vitro (34,35), but no *trmA* gene or *trmF* has been found in still incompletely sequenced genome of this Gram-positive bacterium. This observation raises the possibility that one of the S-AdoMet-dependent rRNA MTase paralogs, which we have detected in non-annotated genome sequence of this species, could act as a tRNA methylase. This idea is further supported by an experimental observation indicating that a *Pyrococcus abyssi* protein highly similar to *E. coli* rRNA:m^5^U MTase *RumA* (13) is actually a site-specific methylase for U-54 in tRNA (J. Urbonavicius, S. Auxilien, K. Trachana and H. Grosjean, unpublished data). We also expect that in many bacteria containing TrmF0, the methylation of U-54 in their tRNAs depends on folate metabolism, while the formation of m^5^U in their rRNA is dependent on S-AdeoMet, as it is in

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**Table 1. Non-exhaustive distribution of the *trmA*^4^ and *trmF* (gid) coding for putative tRNA:m^5^U-54 methyltransferases in bacteria**

| Folate/FAD-dependent pathway | S-AdoMet-dependent pathway | No methylation/unknown |
|-----------------------------|---------------------------|------------------------|
| *trmA* absent/*trmF* present | *trmA* present/*trmF* absent | Both *trmA* and *trmF* absent |
| Organisms of class one      | Organisms of class two    | Organisms of class three |
| Actinobacteria (high GC Gram+) | Proteobacteria             | Actinobacteria (high GC Gram+) |
| *S. therophilum*            | *Beta-proteobacteria*     | *Corynebacteriaceae* |
| *R. xylanophilus*           | *Neisseria meningitis*    | *Corynebacterium glutamicum* |
| Cyanobacteria               | *Neisseria gonorrhoeae*   | *Corynebacterium efficiens* |
| *Synechococcus elongates*   | *Epsilon-proteobacteria*  | *Mycobacteriaceae* |
| *A. variabilis*             | *Campylobacter*            | *Mycoplasma* |
| Firmicutes                  | *Helicobacter hepaticus*  | *Mycoplasma* |
| *Bacillus*                  | *Wolinella succinogenes*  | *Chlamydiae* |
| *B. subtilis*               | *Gamma-proteobacteria*    | *Chlamydiae* |
| *L. lactis*                 | *Acinetobacter*            | *Firmicutes* |
| *B. subtilis*               | *E.coli*                   | *Bacillus* |
| *L. lactis*                 | *Haemophilus*              | *Geobacillus kaustophilus* |
| *S. aureus*                 | *Pseudomonas*              | *G. stearothermophilus* |
| *M. mycoides*               | *P. multocida*             | *Mycoplasma* |
| *M. capricolum*             | *S. flexneri*              | *Mycoplasma* |
| *P. multocida*              | *Vibrio*                   | *Mycoplasma* |
| Thermophiles                | *Y. pseudotuberculosis*    | *Mycoplasma* |
| *A. aeolicus*               | *A. tumefaciens*           | *Proteobacteria* |
| *S. thermophilus*           | *B. pseudofirmus*          | *Alpha-proteobacteria* |
| *M. smegmatis*              | *M. chelonae*              | *Rickettsia* |
| *P. aeruginosa*             | *M. galliseptica*          | *Epsilon-proteobacteria* |
| *B. multivorans*            | *M. mycoides*              | *Helicobacter pylori* |
| *S. flexneri*               | *M. flocculenta*           | *Spirochaetales* |
| *B. vulgai*                 | *S. suis*                  | *Borrelia* |
| *B. Halodurans*             | *M. mycoides*              | *Salmonella* |

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^4 Only sequences with >30% sequence identity with *E. coli* TrmA were considered as TrmA orthologs. These sequences do not contain a sequence motif coordinating iron–sulfur cluster as in ribosomal RNA methyltransferases (12), except for the *Pyrococcus* sp. (see Discussion). Species that are indicated as lacking *trmA* contain genes homologous to *E. coli* *trmA* that likely participate in methylation of rRNA (51).
M. lysodeikticus (51). To construct a more comprehensive evolutionary history for this large family of m^3^U-forming enzymes, as well as other 5-methylpyrimidine MTases, such as those forming m^3^C in RNA and in DNA (52,53), experimental identification of the exact nucleotide target(s) within an RNA for each of these MTases is needed.

In this work, we also considered the possible evolutionary relationship between ribothymidylate synthase TrmFO and the thymidylate synthase ThyX family of enzymes that catalyze a very similar reaction (19). Although both ThyX and TrmFO proteins use flavin (FADH^2^) nucleotide as cofactor, our studies have indicated that they do not belong to the same family of flavoproteins. In particular, TrmFO proteins lack the characteristic conserved residues required for catalysis, substrate and/or cofactor binding of ThyX proteins (54). In addition, the novel FAD binding fold found in ThyX proteins does not show significant similarity to the classical Rossman-fold predicted for TrmFO proteins (21,38). Thus, in the light of this information, a common origin for TrmFO and ThyX proteins appears unlikely. Therefore, direct comparison of the reaction mechanisms between TrmFO and ThyX proteins cannot be done. Our work suggests, for the first time, that the use of CH_2_2(H)_P and FAD in the post-transcriptional methylation of polynucleotides (pre-tRNA) or of a mononucleotide (dUMP) during DNA precursor synthesis has been established independently at least twice during evolution.

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**REFERENCES**

1. Limbach,P.A., Crain,P.F. and McCloskey,J.A. (1994) Summary: the modified nucleosides of RNA. *Nucleic Acids Res.*, 22, 2183–2196.
2. Sprinzl,M. and Vassilenko,K.S. (2005) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.*, 33, D139–D140.
3. Shigii,N., Suzuki,T., Tamakoshi,M., Oshima,T. and Watanebe,K. (2002) Conserved bases in the TpAC loop of tRNA are determinants for thermophile-specific 2-thiouridylate at position 54. *J. Biol. Chem.*, 277, 39128–39135.
4. Fleissner,E. and Borek,E. (1962) A new enzyme of RNA synthesis: RNA methylase. *Proc. Natl Acad. Sci. USA.*, 48, 1199–1203.
5. Svensson,L., Boman,H.G., Eriksson,K.G. and Kjellin,K. (1963) Studies on microbial RNA. I. Transfer of methyl groups from methionine to soluble RNA from *Escherichia coli*. *J. Mol. Biol.*, 16, 254–271.
6. Björk,G.R. (1975) Transductional mapping of gene trmA responsible for the production of 5-methyluracil in ribonucleic acid of *Escherichia coli*. *J. Bacteriol.*, 124, 92–98.
7. ny,T. and Björk,G.R. (1980) Cloning and restriction mapping of the trmA gene coding for transfer ribonucleic acid (5-methyluridyl) methyltransferase in *Escherichia coli* K-12. *J. Bacteriol.*, 142, 371–379.
8. Kealey,J.T., Gu,X. and Santi,D.V. (1994) Enzymatic mechanism of tRNA (m^5^U54)methyltransferase. *Biochemistry*, 34, 1133–1142.
9. Fauman,E.B., Blumenthal,R.M. and Cheng,X. (1999) Structure and evolution of AdoMet-dependent methyltransferases. In Cheng,X. and Blumenthal,R.M. (eds), *S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions*. World Scientific, Singapore, pp. 1–38.
10. Bujnicki,J.M., Droogmans,L., Grosjean,H., Purushothaman,S.K. and Lapeyre,B. (2004) Bioinformatics-guided identification and experimental characterization of novel RNA methyltransferases. In Bujnicki,J.M. (ed.), *Nucleic Acids and Molecular Biology: Practical Bioinformatics series*. Springer-Verlag, Berlin, Heidelberg, Vol. 15, pp. 139–168.
11. Tatusov,R.L., Fedorova,N.D., Jackson,J.D., Jacobs,A.R., Kryutin,B., Koonin,E.V., Krylov,D.M., Mazumder,R., Mekhedov,S.L., Nikolskaya,A.N. et al. (2003) The COG database: an updated version includes eukaryotes. *BMC Bioinformatics*, 4, 41.
12. Agarwalla,S., Kealey,J.T., Santi,D.V. and Stroud,R.M. (2002) Characterization of the 23 S ribosomal RNA m^5^U1939 methyltransferase from *Escherichia coli*. *J. Biol. Chem.*, 277, 8835–8840.
13. Madsen,C.T., Mengel-Jorgensen,J., Kirpekar,F. and Douthwaite,S. (2003) Identifying the methyltransferases for m^5^U747 and m^5^U1939 in 23S rRNA using MALDI mass spectrometry. *Nucleic Acids Res.*, 31, 4738–4746.
14. Delk,A.S., Nagle,D.P. Jr and Rabinowitz,J.C. (1980) Methylenetetrahydrofolate-dependent biosynthesis of ribothymidine in transfer RNA of *Streptococcus faecalis*. Evidence for reduction of the 1-carbon unit by FADH^2_. *J. Biol. Chem.*, 255, 4387–4390.
15. Samuel,C.E. and Rabinowitz,J.C. (1974) Initiation of protein synthesis by folate-sufficient and folate-deficient *Streptococcus faecalis* R: partial purification and properties of methionyl-transfer ribonucleic acid synthetase and methionyl-transfer ribonucleic acid formyltransferase. *J. Bacteriol.*, 118, 21–31.
16. Arnold,H.H. and Kersten,H. (1975) Inhibition of the tetrahydrofolate-dependent biosynthesis of ribothymidine in rRNAs of *B.subtilis* and *M lysodeikticus* by trimethoprim. *FEBS Lett.*, 53, 258–261.
17. Delk,A.S., Romeo,J.M., Nagle,D.P., Jr and Rabinowitz,J.C. (1976) Biosynthesis of ribothymidine in the transfer RNA of *Streptococcus faecalis* and *Bacillus subtilis*. A methylation of RNA involving 5,10-methylene tetrahydrofolate. *J. Biol. Chem.*, 251, 7649–7656.
18. Delk,A.S., Nagle,D.P., Jr, Rabinowitz,J.C. and Straub,K.M. (1979) The methylene tetrahydrofolate-mediated biosynthesis of ribothymidine in the transfer-RNA of *Streptococcus faecalis*: incorporation of hydrogen from solvent into the methyl moiety. *Biochem. Biophys. Res. Commun.*, 86, 244–251.
19. Myllykallio,H., Lipowski,G., Leduc,D., Filee,J., Forterre,P. and Liebl,U. (2002) An alternative flavin-dependent mechanism for thymidylate synthesis. *Science*, 297, 105–107.
20. Gilardi,M., Bitan-Banin,G., Mevarech,M. and Ortenberg,R. (2002) Genetic evidence for a novel thymidylate synthase in the halophilic archaeon *Halobacterium salinarum* and in *Campylobacter jejuni*. *FEMS Microbiol. Lett.*, 216, 105–109.
21. Mathews,I., Deacon,A.M., Canaves,J.M., McMullan,D., Lesley,S.A., Agarwalla,S. and Kuhn,P. (2003) Functional analysis of substrate and cofactor complex structures of a thymidylate synthase-complementing protein. *Structure (Camb.)*, 11, 677–690.
22. Finer-Moore,J.S., Santi,D.V. and Stroud,R.M. (2003) Lessons and conclusions from dissecting the mechanism of a bisubstrate enzyme: thymidylate synthase mutagenesis, function, and structure. *Biochemistry*, 42, 248–256.
23. Agarwalla,N., Lesley,S.A., Kuhn,P. and Cohen,A. (2004) Mechanistic studies of a flavin-dependent thymidylate synthase. *Biochemistry*, 43, 10295–10301.
24. Graziani, S., Xia, Y., Gurnon, J.R., Van Eten, J.L., Leduc, D., Skoulozouros, S., Myllykallio, H. and Liebl, U. (2004) Functional analysis of FAD-dependent thymidylate synthase ThyX from *Paramecium bursaria* Chlorovirus-1. *J. Biol. Chem.*, **279**, 54340–54347.

25. Grattis, S.G. and Palfey, B.A. (2005) Direct observation of the participation of flavin in product formation by thyX-encoded thymidylate synthase. *J. Am. Chem. Soc.*, **127**, 832–833.

26. Griffin, J., Roschik, C., Iliffe-Lee, E. and McClarty, G. (2005) Catalytic mechanism of *Chlamydia trachomatis* flavin-dependent thymidylate synthase. *J. Biol. Chem.*, **280**, 5456–5467.

27. Perret, V., García, A., Puglisi, J., Grosjean, H., Ebelt, J.P., Florentz, C. and Giege, R. (1990) Conformation in solution of yeast tRNA(Asp) transcripts deprived of modified nucleotides. *Biochemistry*, **32**, 735–743.

28. Grosjean, H., Droogmans, L., Giege, R. and Uhlenbeck, O.C. (1990) Guanosine modifications in runoff transcripts of synthetic transfer RNA-Phe genes microinjected into Xenopus oocytes. *Biochim. Biophys. Acta*, **1050**, 267–273.

29. Grosjean, H., Keith, G. and Droogmans, L. (2004) Detection and quantification of modified nucleotides in RNA using thin-layer chromatography. *Methods Mol. Biol.*, **265**, 357–391.

30. Bujnicki, J.M., Oudjama, Y., Roovers, M., Owczarek, S., Caillet, J. and Droogmans, L. (2004) Identification of a bifunctional enzyme MnmC involved in the biosynthesis of a hypermodified uridine in the wobble position of tRNA. *RNA*, **10**, 1236–1242.

31. von Ehrenstein, G. (1967) Isolation of RNA from intact *Escherichia coli* cells. *Meth. Enzymol.*, **12**, 588–596.

32. Gehrke, C.W. and Kuo, K.C. (1989) Ribonucleoside analysis by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, **471**, 3–36.

33. Delk, A.S., Nagle, D.P. Jr and Rabinowitz, J.C. (1979) Purification of methyltetrahydrofolate-dependent methyltransferase catalyzing biosynthesis of ribothymidine in transfer RNA of *Streptococcus faecalis*. In Kisluk, R.L. and Brown, G.M. (eds), *Chemistry and Biology of Pteridines*. Elsevier/North Holland Publishing, NY, pp. 389–394.

34. Agris, P.F., Koh, H. and Soll, D. (1973) The effect of growth temperatures on the in vivo ribose methylation of Bacillus stearothermophilus transfer RNA. *Arch. Biochem. Biophys.*, **154**, 277–282.

35. Schmidt, W., Arnold, H.H. and Kersten, H. (1977) Tetrahydrofolate-dependent biosynthesis of ribothymidine in transfer ribonucleic acids of Gram-positive bacteria. *J. Bacteriol.*, **129**, 15–21.

36. Nordlund, M.E., Johansson, J.O., von Pawel-Rammingen, U. and Bystrom, A.S. (2000) Identification of the TRM2 gene encoding the tRNA(m5U54)methyltransferase of *Saccharomyces cerevisiae*. *RNA*, **6**, 844–860.

37. Constantinescu, F., Motorin, Y. and Grosjean, H. (1999) Transfer RNA modification enzymes from *Pyrococcus furiosus*: detection of the enzymatic activities in vitro. *Nucleic Acids Res.*, **27**, 1308–1315.

38. Dym, O. and Eisenberg, D. (2001) Sequence-structure analysis of FAD-containing proteins. *Protein Sci.*, **10**, 1712–1728.

39. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.

40. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, **25**, 4876–4882.

41. Bjork, G.R. and Isaksson, L.A. (1970) Isolation of mutants of *Escherichia coli* lacking 5-methyluracil in transfer ribonucleic acid or 1-methylguanine in ribosomal RNA. *J. Mol. Biol.*, **51**, 83–100.

42. White, D.J., Merod, R., Thomasson, B. and Hartzell, P.L. (2001) GidA is an FAD-binding protein involved in development of *Myxococcus xanthus*. *Mol. Microbiol.*, **42**, 503–517.

43. Iwasaki, W., Miyatake, H., Ebihara, A. and Miki, K. (2004) Crystallization and preliminary X-ray crystallographic studies of the small form of glucose-inhibited division protein A from *Thermus thermophilus* HB8. *Acta Crystallogr. D Biol. Crystallogr.*, **60**, 515–517.

44. Colby, G., Wu, M. and Tzagoloff, A. (1998) MTO1 codes for a mitochondrial protein required for respiration in paromomycin-resistant mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **273**, 27945–27952.

45. Breeze, D., Colot, V., Radman, M. and Taadei, F. (2001) Translation misreading: a tRNA modification counteracts a +2 ribosomal frameshift. *Genes Dev.*, **15**, 2295–2306.

46. Li, R., Li, X., Yan, Q., Qin, M.J. and Guan, M.X. (2003) Identification and characterization of mouse MTO1 gene related to mitochondrial tRNA modification. *Biochim. Biophys. Acta*, **1629**, 53–59.

47. Scrima, A., Vetter, I.R., Armengod, M.E. and Wittinghofer, A. (2005) The structure of the TrmE GTP-binding protein and its implications for tRNA modification. *EMBO J.*, **24**, 23–33.

48. Umeda, N., Suzuki, T., Ohya, Y., Shibano, H. and Watanabe, K. (2005) Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. *Implications for the molecular pathogenesis of human mitochondrial diseases. J. Mol. Biol.*, **300**, 1613–1624.

49. Andachi, Y., Yamao, F., Muto, A. and Osawa, S. (1989) Codon recognition patterns as deduced from sequences of the complete set of transfer RNA species in *Mycoplasma capricolum*. Resemblance to mitochondria. *J. Mol. Biol.*, **209**, 37–54.

50. Johnson, L., Hayashi, H. and Soll, D. (1970) Isolation and properties of a transfer ribonucleic acid deficient in ribothymidine. *Biochemistry*, **9**, 2823–2831.

51. Schmidt, W., Arnold, H.H. and Kersten, H. (1975) Biosynthetic pathway of ribothymidine in *B.subtilis* and *M.lysedeticus* involving different coenzymes for transfer RNA and ribosomal RNA. *Nucleic Acids Res.*, **2**, 1043–1051.

52. Anantharaman, V., Koonin, E.V. and Aravind, L. (2002) Comparative genomics and evolution of proteins involved in RNA metabolism. *Nucleic Acids Res.*, **30**, 1427–1464.

53. Bujnicki, J.M., Feder, M., Ayres, C.L. and Redman, K.L. (2004) Sequence-structure-function studies of tRNA(m5C) methyltransferase Trm4p and its relationship to DNA(m5C) and RNA(m5U) methyltransferases. *Nucleic Acids Res.*, **32**, 2453–2463.

54. Leduc, D., Graziani, S., Lipowski, G., Marchand, C., Le Marechal, P., Liebl, U. and Myllykallio, H. (2004) Functional evidence for active site location of tetrameric thymidylate synthase ThyX at the interphase of three monomers. *Proc. Natl Acad. Sci. USA*, **101**, 7252–7257.

55. Hall, R.H. (1971) The modified nucleosides in nucleic acids. Columbia University Press, NY and London.