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# Identification of a novel gene encoding a flavin-dependent tRNA:m$^5$U 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 tRNA methyltransferases (tRNA:m$^5$U-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*$^5$, *N*$^{10}$-methylenetetrahydrofolate ($CH_2H_4$folate). We have identified the gene for *Bacillus subtilis* tRNA:m$^5$U-54 MTase. The encoded recombinant protein contains tightly bound flavin and is active in *Escherichia coli* mutant lacking m$^5$U-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$^5$U, 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$^5$s$^2$U or s$^2$T, reviewed in (3)], while in certain Eukarya, a 2′-*O*-methyl-derivative is occasionally found [m$^5$Um (2)].

Site-specific methylation of U-54 in *Escherichia coli* tRNA is catalyzed by tRNA:m$^5$U-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$^5$U-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$^5$U 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$^5$U-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*$^5$, *N*$^{10}$-methylenetetrahydrofolate ($CH_2H_4$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$^5$U-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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$m^5U$-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 $CH_2H_4$folate 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 $CH_2H_4$folate 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 methylating nucleotides in tRNA and DNA precursor using $CH_2H_4$folate 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 *gid*Ω*erm*$^R$ gene was kindly provided by S. Seror [European functional analysis project of *B.subtilis* (http://bacillus.genome.jp/bsorf-bin/BSORF_data_view.pl?ACCESSION=BG11008)]. *E.coli* strain GRB113 (*metA*, *trmA5*, *zij-90*::Tn*10*), encoding an inactivated TrmA protein was a kind gift from G. R. Björk, Umeå University, Sweden. *E.coli* Sure® strain (e14$^-$(McrA$^-$) Δ*mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::*Tn*5* (Kan$^r$) *uvrC* [F′ *proAB lacI*$^q$ZΔ*M15* Tn*10* (Tet$^r$)] was purchased from Stratagene.

### Construction of an N-terminal $His_6$-tagged $_{Bsu}$GidA and $_{Bsu}$Gid-overexpressing plasmids and purification of the corresponding recombinant proteins

The *gidA* (GIDA_BACSUB; P25812) and *gid* genes (GID_BACSU; P39815; renamed *trmFO* 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 (CGGGATCCatggggtatgaagcaggccaatac) and gidArev (TCCCCCGGGctactcggctatcttcgcaatgcg) or gidfw (CGGGATCCatgaaccaacaaacagtgaatgta) and gidrev (TCCCCCGGGctatattgttttcgaaattgtttg). The resulting 1893 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® strain, and pQE80L-BsuGid was transformed into Sure® or GRB113 (*trmA5*) strain. Resulting strains were grown at 37°C in 500 ml of Luria–Bertani (LB) medium (Invitrogen) containing 100 μl/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 were flash-frozen in liquid $N_2$ and stored at −80°C. Frozen cells were thawn 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 (*gid*Ω*erm*$^R$) 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-$_{Bsu}$Gid/GRB113 (*trmA5*) and pQE80L/GRB113 (*trmA5*) were prepared similarly as for the protein purification, except that they were grown at 37°C to an $OD_{600}$ of 0.8 in 10 ml of liquid Luria Broth with 100 μl/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-$^{32}$P]UTP-labeled yeast tRNA$^{Asp}$ transcript, used for determining the tRNA:m$^5$U-54 MTase activity of TrmFO (Gid) protein, was prepared and purified as described elsewhere (27,28). A total of 50–100 fmol of [$^{32}$P]-labeled tRNA$^{Asp}$ 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)-$N^5$,$N^{10}$-$CH_2H_4$PteGlu-$Na_2$ (methylenetetrahydrofolate, 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 nucleotides were separated by 2D thin-layer chromatography (2D-TLC) and data were analyzed as described previously (29). Methylating activity of purified recombinant $_{Bsu}$Gid (TrmFO) (1 µg per test) and $_{Bsu}$GidA protein (1 µg 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 (*gid*Ω*erm*$^R$) 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 $NH_4Cl$, 62 µM [*methyl*-14C]-AdoMet (53 Ci/mol, 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 (*gid*Ω*erm*$^R$) 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* GRB113 (*trmA5*), transformed by pQE80L-$_{Bsu}$Gid 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 $OD_{600}$ = 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 $LC_{18}$ 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 (m$^5$U-54) of tRNA using $CH_2H_4$folate 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.lysodeikticus* 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:m$^5$U-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-L-A-G-[TS]-E-X-A, see details below) 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:m$^5$U-54 MTase (33).

### COG1206 proteins have a wide phylogenetic distribution

Systematic screening of >200 fully sequenced genomes (http://www.ncbi.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 archaeal 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 m$^5$U-54 formation in tRNA

To determine whether Gid proteins are involved in the biosynthesis of m$^5$U-54, the presence of this methylated nucleoside was analyzed in *B.subtilis* tRNA isolated from a BFS2838 (*gid*Ω*erm*$^R$) strain, lacking functional Gid protein (kindly provided by S. Seror, University of Paris XI).

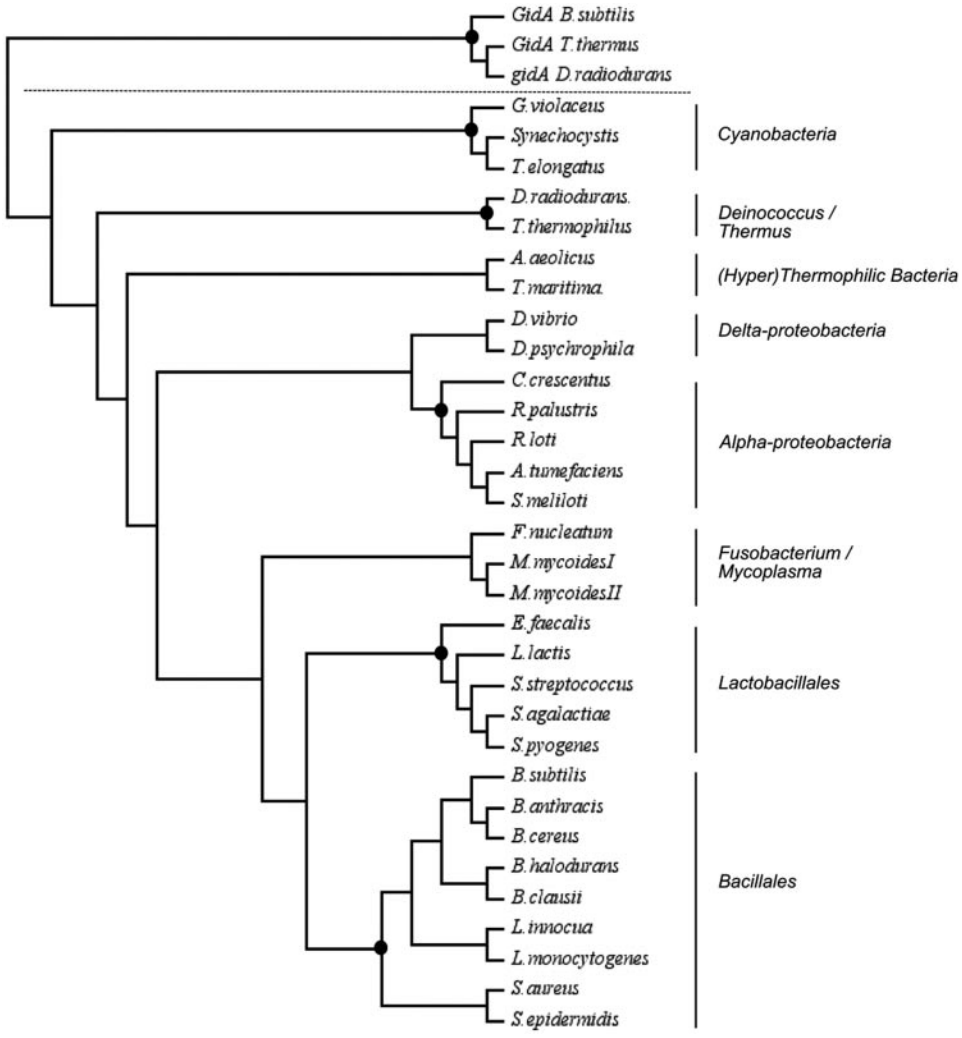



**Figure 1.** A phylogenetic tree based on a subset of Gid homologs retrieved by pattern-hit initiated BLAST algorithm. Clustal X was used for sequence alignments and phylogenetic trees were constructed using the neighbor-joining methods. GidA sequences from *B.subtilis*, *T.thermophilus* and *Deinococcus radiodurans* were used as an outgroup. Branch points with closed circles indicate a bootstrap support >90%. The shown topology was also supported by quartet puzzling with maximum likelihood analysis performed using Tree-Puzzle 5.1 program implemented at www.pasteur.fr (data not shown).

No obvious phenotype has been described for this *B.subtilis* strain (see 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 *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 $m^5U$ is absent in the tRNA from the *gid*Ω*erm*$^R$ mutant, whereas tRNA of the wild-type strain 168 contains the $m^5U$ modification. The small residual peak eluting at the same position as $m^5U$ 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 *gid*Ω*erm*$^R$ 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 $tRNA^{Asp}$ gene was used as substrate, and incubations were performed in the presence of $CH_2H_4$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 $m^5U$-54 *in vitro*, the extract from the *gid*Ω*erm*$^R$ mutant strain did not catalyze such a methylation reaction, thus indicating that the *gid* gene product is involved in the production of $m^5U$-54 in tRNA.

## The Gid protein of *B.subtilis* is sufficient for methylation of U-54 in *E.coli* tRNA *in vivo*

To investigate whether the *B.subtilis* Gid protein alone can substitute for the S-AdoMet-dependent *E.coli* TrmA protein for the formation of $m^5U$-54 *in vivo*, we cloned the *B.subtilis gid* into an *E.coli* expression vector pQE80L, under the control of an IPTG inducible promoter. The resulting plasmid, pQE80L-$_{Bsu}$Gid was transformed into an *E.coli* strain

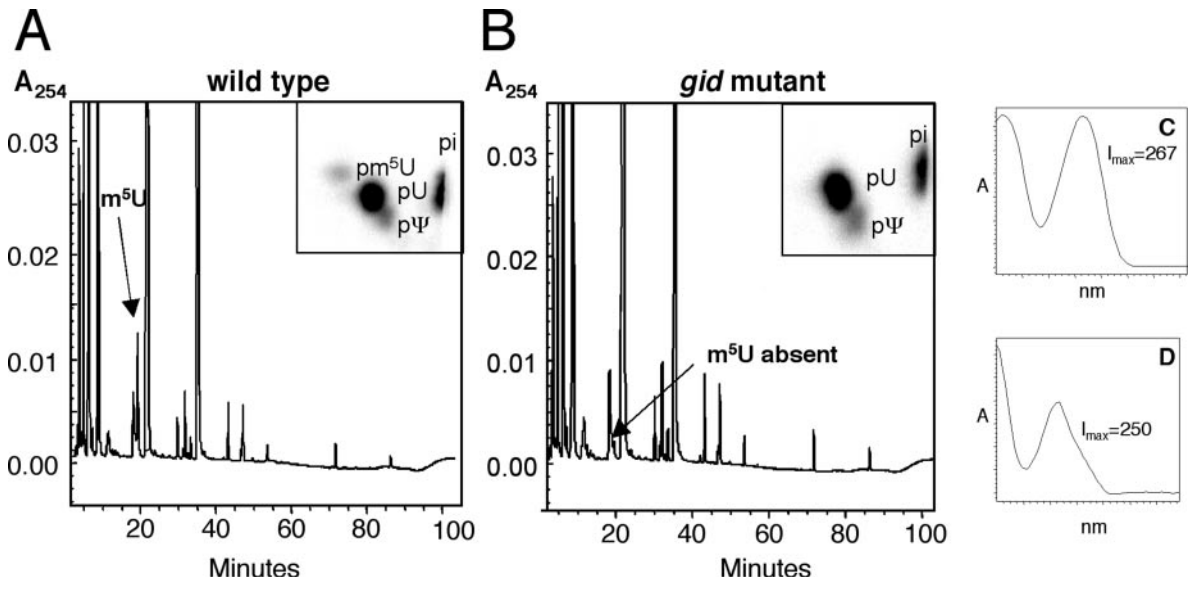



**Figure 2.** *B.subtilis* BFS2838 (*gid*Ω*erm*$^{R}$) strain lacks m$^5$U modification in the tRNA. Bulk tRNAs from wild-type *B.subtilis* 168 (**A**) or the *gid*Ω*erm*$^{R}$ mutant BFS2838 (**B**) were isolated, completely digested to nucleosides by nuclease P1 and alkaline phosphatase and analyzed by HPLC (see Materials and Methods). Alternatively, T7 polymerase transcripts of yeast wild-type tRNA$^{Asp}$, uniformly labeled with [α-$^{32}$P]UTP were incubated for 1 h at 37°C with an S100 cell extract from 168 [inset in (A)] or BFS2838 [*gid*Ω*erm*$^{R}$, inset in (B)]. After incubation, bulk tRNAs were completely digested to monophosphate nucleosides by nuclease P1 and analyzed by 2D-TLC. Radiolabeled compounds were detected and quantified using PhosphoImager detector. (**C**) The spectrum analysis of the HPLC fraction corresponding to m$^5$U (A). (**D**) The spectrum of the small peak detected in (B), corresponding to inosine. Nature of the modified nucleosides in chromatography peaks was determined by comparison with the standards (55).

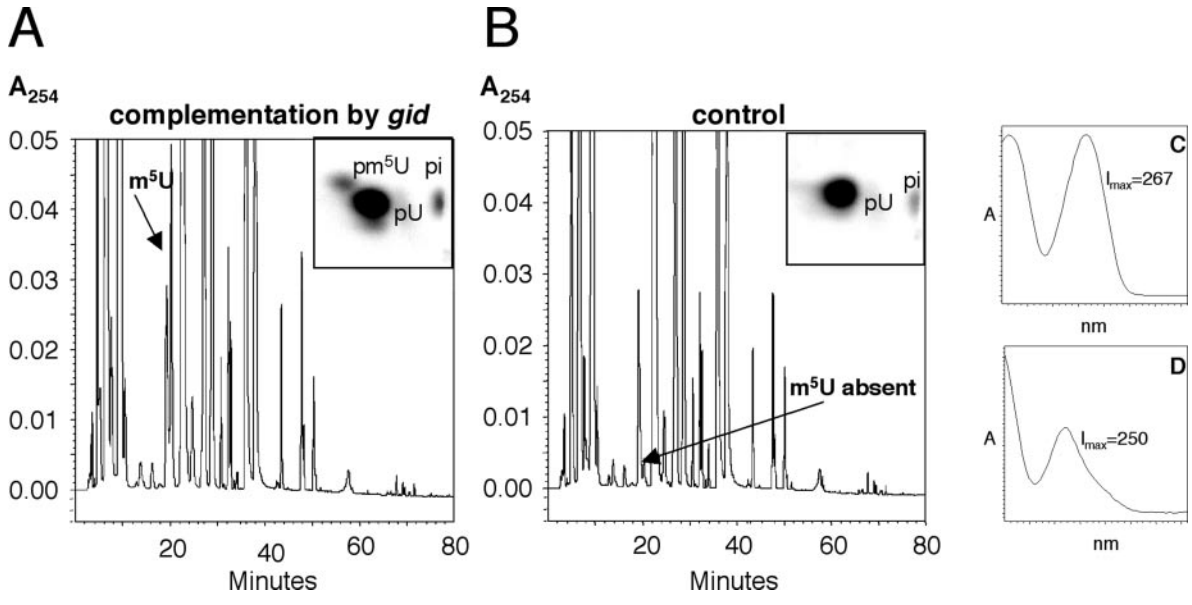
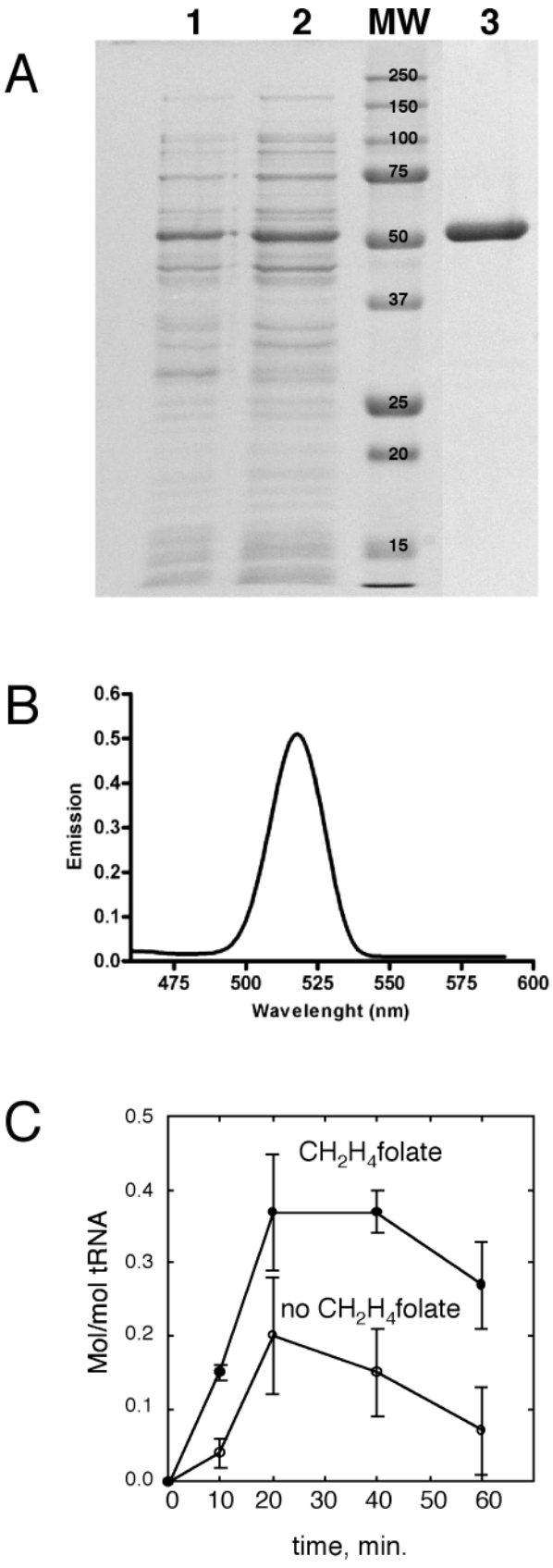



**Figure 3.** Recombinant $_{Bsu}$Gid protein catalyzes the formation of the m$^5$U-54 modification in tRNA *in vivo*. HPLC analysis performed with bulk tRNAs purified from *E.coli* GRB113 (*trmA*5) transformed with pQE80L-$_{Bsu}$Gid (**A**), or with the 'empty plasmid' pQE80L (**B**). Insets in (A) and (B) correspond to autoradiograms of tRNA hydrolysates resulting from *in vitro* enzymatic activity tests performed with cell-free extracts of *E.coli* GRB113 (*trmA*5) strain transformed with pQE80L-$_{Bsu}$Gid [inset (A)] or with pQE80L [inset (B)]. As in Figure 2, (**C**) shows the spectrum analysis of the HPLC fraction corresponding to m$^5$U (A). (**D**) The spectrum of the small peak detected in (B), which corresponds to inosine.

GRB113, carrying *trmA*5 mutation. This *E.coli* strain grows normally in LB medium but completely lacks S-AdoMet-dependent tRNA:m$^5$U-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$^5$U 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$^5$U 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 $m^5U$ 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 $m^5U$). In parallel, the S10 cell extract was incubated together with appropriate cofactors and [UTP-$^{32}$P] labeled yeast $tRNA^{Asp}$ transcript. The P1-hydrolyzate of the resulting modified tRNA was then analyzed by 2D-TLC. Results in Figure 3A and B (insets) indicate that $m^5U$-54 in $tRNA^{Asp}$ is formed only when a cell-free extracts from the *E.coli trmA5* strain transformed with pQE80L-$_{Bsu}$Gid, confirming that the *B.subtilis* Gid protein efficiently modified tRNAs under the physiological conditions of *E.coli* cells.

### Purified recombinant $_{Bsu}$Gid protein catalyzes the *in vitro* formation of $m^5U$-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 pQE80L-$_{Bsu}$Gid. 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 $m^5U$-54 in [$^{32}$P]-labeled yeast $tRNA^{Asp}$ 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 $_{Bsu}$Gid 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-$_{Bsu}$Gid/SURE; lane 2, S10 fraction from *E.coli* pQE80L-$_{Bsu}$Gid/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 $_{Bsu}$Gid protein. The observed emission maximum (after excitation at 450 nm) at 520 nm is typical for flavin nucleotides. (**C**) Time course of $m^5U$-54 formation catalyzed by $_{Bsu}$Gid. The molar ratio of $m^5U$ over total U in yeast $tRNA^{Asp}$ was evaluated over time at 37°C in the presence (closed circles) or absence (open circles) of $CH_2H_4$folate.

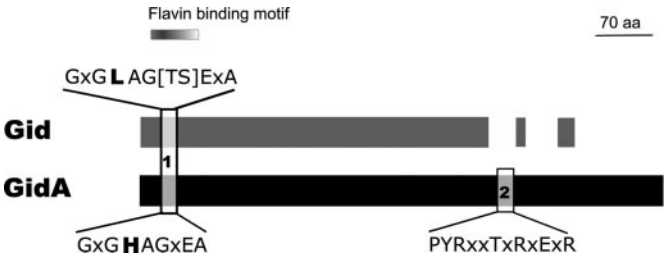



**Figure 5.** Comparison of the amino acid sequences of Gid (TrmFO) and GidA proteins. GidA proteins are systematically longer than Gid proteins, having an additional C-terminal domain. Both proteins apparently bind FAD cofactors (42,47) (data not shown). The two proteins can be discriminated by a sequence motif partially overlapping with the FAD binding motif ('motif 1'). An additional GidA-specific motif located at the C-terminus of the protein also distinguishes the two paralogs ('motif 2').

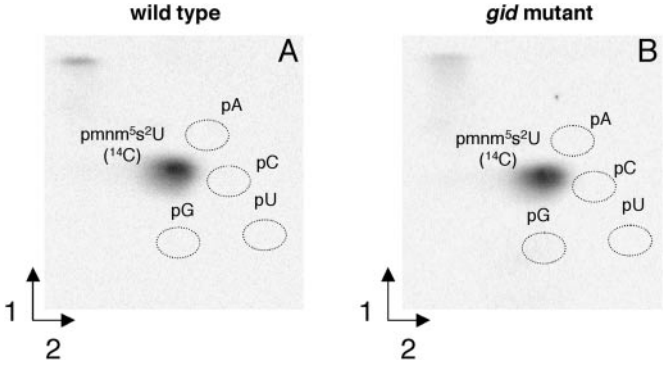



**Figure 6.** tRNAs from the *gidΩerm*$^R$ mutant are substrates for the MnmC protein. (**A** and **B**) correspond to autoradiograms of a TLC analysis of nuclease P1-hydrolysate of bulk tRNAs, isolated from *B.subtilis* strain 168 (A) or from BFS2838 (*gidΩerm*$^R$) mutant (B), previously incubated with purified recombinant *E.coli* MnmC and [*methyl*-$^{14}$C]-labeled AdoMet (see Materials and Methods) (30). Radiolabeled compounds were detected and quantified after 3 days exposure using a PhosphoImager. Results indicated that both bulk tRNAs are equally well methylated by MnmC showing that in both the cases, cmnm$^5$U-34 was prevalent in the tRNAs.

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

TrmFO proteins of ≈50 kDa (designated Gid in Genome Data Banks) show ∼40% sequence similarity with another protein family referred to as GidA proteins (in reference to *E.coli* protein of ≈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 *Mycobacterium* and *Corynebacterium* species (http://string.embl.de/).

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-carboxymethylaminomethyl-uridine (cmnm$^5$U) and 5-methylaminomethyl-uridine (mnm$^5$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 (*gidΩerm*$^R$) 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 (*gidΩerm*$^R$), to become fully modified *in vitro* into [$^{14}$C]mnm$^5$s$^2$U-34 upon incubation with purified recombinant MnmC protein of *E.coli*. 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 nm$^5$s$^2$U-34 and further methylates it into mnm$^5$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 mnm$^5$s$^2$U-34 occurs equally well in the tRNAs of both the wild-type *B.subtilis* strain and the *gidΩerm*$^R$ 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 $CH_2H_4$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$^5$U-54 formation in tRNA of certain organisms, $N^5$, $N^{10}$-methylenetetrahydrofolate, 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 tRNA 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 $m^5U$ in rRNA as in the case of the S-AdoMet-dependent pathway (12,13). Strikingly, the folate-dependent TrmFO 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 enterobacteriacae (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 *trmFO* ortholog. In contrast, orthologs of *trmFO* 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 *Mycoplasma* species, seemingly lack either *trmFO* or *trmA* genes, suggesting that the uridine at position 54 in their 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 *trmFO* alleles whose functional role is unclear and is worth investigation. In contrast, thermophilic *G.stearothermophilus* displays an S-AdoMet-dependent activity for $m^5U$ modification *in vitro* (34,35), but no *trmA* gene or *trmFO* has been found in still uncompletely 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:$m^5U$ 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 TrmFO, the methylation of U-54 in their tRNAs depends on folate metabolism, while the formation of $m^5U$ in their rRNA is dependent on S-AdoMet, as it is in

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

| Folate/FAD-dependent pathway<br>*trmA* absent/*trmFO* present<br>Organisms of class one | S-AdoMet-dependent pathway<br>*trmA* present/*trmFO* absent<br>Organisms of class two | No methylation/unknown<br>Both *trmA and trmFO* absent<br>Organisms of class three |
|---|---|---|
| Actinobacteria (high GC Gram+) | Proteobacteria | Actinobacteria (high GC Gram+) |
| *Symbiobacterium thermophilum* | Beta-proteobacteria | *Corynebacteriaceae* |
| *Rubrobacter xylanophilus* | *Neisseria meningitis* | *Corynebacterium glutamicum* |
| | *Neisseria gonorrhoeae* | *Corynebacterium efficiens* |
| Cyanobacteria | Epsilon-proteobacteria | Mycobacteriaceae |
| *Synechococcus elongates* | *Campylobacter* sp. | *Mycobacterium* sp. |
| *Anabaens variabilis* | *Helicobacter hepaticus* | *Mycoplasma* sp. |
| | *Wolinella succinogenes* | |
| Firmicutes | Gamma-proteobacteria | Chlamydiales |
| Bacillales | *Acinetobacter* sp. | *Chlamydiae* sp. |
| *Bacillus* sp. | *E.coli* | |
| *Listeria* sp. | *Haemophilus* sp. | Firmicutes |
| *Staphylococcus* sp. | *Pseudomonas* sp. | *Bacillales* |
| Lactobacillales | *Pasteurella multocida* | *Geobacillus kaustophilus* |
| *Lactobacillus* sp. | *Salmonella* sp. | *G.stearothermophilus* |
| *Streptococcus* sp. | *Shigella flexneri* | *Mycoplasmatales* |
| Mycoplasmatales | *Vibrio* sp. | *Mycoplasma* sp. |
| *Mycoplasma mycoides* | *Yersinia pseudotuberculosis* | |
| Proteobacteria | | Proteobacteria |
| Alpha-proteobacteria | | Alpha-proteobacteria |
| *Rhizobial* sp. | Probably all Eukarya | *Rickettsia* sp. |
| *Rhodobacter* sp. | | Epsilon-proteobacteria |
| *Brucella* sp. | Archaea | *Helicobacter pylori* |
| *Agrobacterium tumefaciens* | *Pyrococcus* sp. | |
| Delta-proteobacteria | | Spirochaetales |
| *Geobacter* sp. | | *Borrelia* sp. |
| *Desulfovibrio* sp. | | *Leptospira interrogans* |
| Thermophilic Bacteria | | |
| *Aquifex aeolicus* | | |
| *Thermotoga maritime* | | |
| *T.thermophilus* | | |

[a]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 $m^5U$-forming enzymes, as well as other 5-methylpyrimidine MTases, such as those forming $m^5C$ 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 $CH_2H_4$folate 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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