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The *yggH* Gene of *Escherichia coli* Encodes a tRNA (m^{7}G46) Methyltransferase

Lara G. S. De Bie,[1] Martine Roovers,[1] Yamina Oudjama,[2] Ruddy Wattiez,[3] Catherine Tricot,[2] Victor Stalon,[1,2] Louis Droogmans,[1*] and Janusz M. Bujnicki[4]

Laboratoire de Microbiologie, Université Libre de Bruxelles,[1] and Institut de Recherches Microbiologiques Jean-Marie Wiame,[2] B-1070 Brussels, and Laboratoire de Chimie Biologique, Université de Mons-Hainaut, B-7000 Mons,[3] Belgium, and Bioinformatics Laboratory, International Institute of Molecular and Cell Biology, 02-109 Warsaw, Poland[4]

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We cloned, expressed, and purified the *Escherichia coli* YggH protein and show that it catalyzes the *S*-adenosyl-L-methionine-dependent formation of *N*^{7}-methylguanosine at position 46 (m^{7}G46) in tRNA. Additionally, we generated an *E. coli* strain with a disrupted *yggH* gene and show that the mutant strain lacks tRNA (m^{7}G46) methyltransferase activity.

About 30 different modified nucleosides have been identified in *Escherichia coli* tRNA. Methylation is one of the most common modifications, and several mutants affected in tRNA methylation have been obtained (5). However, only a few *E. coli* tRNA methyltransferase (MTase) genes have been cloned and characterized: *trmA*, *trmD*, and *trmH* are involved in the formations of m^{5}U54, m^{1}G37, and Gm18, respectively (8, 15, 16). Several tRNA MTases have been purified, and the corresponding genes have been mapped on the *E. coli* chromosome (5, 13), but it has not been convincingly shown which open reading frame (ORF) encodes a given enzyme. On the other hand, evolutionary relationships among various RNA MTase families have been studied and predictions of novel specificities for uncharacterized ORFs have been made (3). Nevertheless, there are still missing links between many known enzymatic activities and predicted RNA MTase genes.

As part of a large-scale project aimed at the identification and classification of novel RNA MTases among the uncharacterized or putative proteins in sequence databases, we analyzed the product of the *E. coli yggH* ORF. This protein exhibits similarity to *S*-adenosyl-L-methionine (AdoMet)-dependent MTases in the predicted cofactor-binding region but shares no specific amino acid signatures with other families of RNA MTases in the predicted catalytic region, suggesting that it may encode an RNA MTase with a novel specificity. Thus, we selected it for experimental characterization.

Amplification and cloning of the *yggH* ORF. The *yggH* ORF was PCR amplified from *E. coli* genomic DNA (strain XL1-Blue) by using *Pfu* DNA polymerase (Promega). The primers (Table 1) were designed to amplify the *yggH* ORF with its ribosome binding site. Primers LDB1 and LDB3 were used for the production of a recombinant YggH protein bearing a C-terminal His tag (YggHH6). Primers LDB1 and LDB2 were used for the production of the untagged YggH.

The PCR products were cloned into the pCR-BluntII-TOPO vector (Invitrogen) according to the manufacturer's instructions, generating the pCR-*yggHH6* and pCR-*yggH* plasmids (the strains and plasmids used are shown in Table 2). The *Xba*I/*Xho*I insert of the pCR-*yggHH6* plasmid was subcloned into the corresponding sites of the pET30b overexpression vector (Novagen), generating the pET-*yggHH6* plasmid.

Expression and purification of the YggHH6 recombinant protein. The YggHH6 protein was expressed in *E. coli* strain BL21(DE3). Transformed cells were grown at 37°C in Luria broth (supplemented with kanamycin at 30 μg/ml) to an optical density at 660 nm of 0.7. At this stage, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM to induce recombinant protein expression. Cells were harvested after 3 h of incubation at 37°C, resuspended in buffer A (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10% glycerol), and lysed by sonication. The lysate was cleared by centrifugation (20,000 × *g* for 10 min) and was applied to a column of Chelating Sepharose Fast Flow (Pharmacia Biotech) charged with Ni^{2+}. The column was washed with buffer A supplemented with 5 mM imidazole, and the adsorbed material was eluted with a linear gradient (0.05 M up to 0.4 M) of imidazole. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At this stage, the YggHH6 preparation contained several minor contaminants (data not shown).

YggHH6 was further purified by gel filtration chromatography. The partially purified enzyme was dialyzed against buffer A supplemented with 200 mM imidazole to keep the protein soluble and was applied on a Superdex 200 column (Pharmacia Biotech) equilibrated with the same buffer. SDS-PAGE analysis of the fractions containing YggHH6 showed two discrete bands (Fig. 1A), both of which corresponded to the YggH protein as demonstrated by mass spectrometry fingerprint analysis. A similar mass fingerprint was obtained for both bands, except for the C-terminal tag tryptic peptide, which was absent for the lower band (result not shown). Thus, the lower band most probably corresponds to a degradation product of YggHH6, lacking the C-terminal His tag. Gel filtration chromatography revealed that the apparent molecular mass of the

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Université Libre de Bruxelles, Institut de Recherche Microbiologiques J.-M. Wiame, 1, avenue E. Gryson, B-1070 Brussels, Belgium. Phone: 32 2 526 7254. Fax: 32 2 526 7273. E-mail: louisd@dbm.ulb.ac.be.

TABLE 1. Oligonucleotides

Oligonucleotide	Sequence	Target gene
LDB1	CCTCTAGAAATTAAGAAGGAGATATACATATGAAAAACGACGTCATTTCACCGG	*yggH*
LDB2	GGCCTCGAGTTATTATTTCACCCTCTCGAAC	*yggH*
LDB3	GGCCTCGAGTTTCACCCTCTCGAACATTAAG	*yggH*
LDB4	CGCCCCGCAACGCCGATAAGGTATC	*metT*
LDB5	ATCTGGTGCGTCTACCAATTTCGCC	*metT*
LDB6	CGCTAATACGACTCACTATAGGCTACGTAGCTCAGTTGGTTAGAG	*metT*
LDB7	CCTGGTGGCTACGACGGGATTCGAACCTGTGAC	*metT*
LDB8	CATAATGATGGGATCACAGGTTCGAATC	*metT*
LDB9	GATTCGAACCTGTGATCCCATCATTATG	*metT*
LDB10	ATGAAAAACGACGTCATTTCACCGGAATTTGATGAAAACGCATTCAAATATGTATCCGCTC	*bla*
LDB11	TACTCCGTGACCAAGACGATGACCACGTTGTTCAAATTTCAGAGTTGGTAGCTCTTGATC	*bla*
LDB12	GCGACGCTTGCATGGTC	*yggH* promoter
LDB13	ACCTGAACGATACGGCG	*yggH*
LDB14	TGTTGAGATCCAGTTCG	*bla*

YggHH6 protein is about 27 kDa. This shows that the protein exists as a monomer.

***yggH* encodes an MTase responsible for the formation of m^7G46 in the variable loop of tRNA.** To determine whether the product of the *yggH* ORF was an MTase acting on tRNA, the purified YggHH6 protein was incubated with ^{14}C-radiolabeled AdoMet (*S*-adenosyl-L-[*methyl*-^{14}C]methionine) and total tRNA was extracted from a methionine-starved P4X-SB25 strain (an *E. coli met* mutant *relA* strain). After incubation, the tRNA was hydrolyzed by nuclease P1 and the resulting nucleotides were analyzed by bidimensional cellulose thin-layer chromatography (2D-TLC) followed by autoradiography. The result shown in Fig. 1B revealed the formation of a single radioactive compound with migration characteristics similar to those of 7-methylguanosine 5′-phosphate (pm^{7}G). Examination of the tRNA sequence database (http://www.uni-bayreuth.de/departments/biochemie/trna/) revealed that m^{7}G is found only at position 46 (in the variable loop) in 23 *E. coli* tRNA species, including $tRNA^{Met}_m$ (Fig. 2A). Therefore, an in vitro transcribed $tRNA^{Met}_m$ was tested as a substrate for the purified YggHH6 protein. The *metT* gene, encoding $tRNA^{Met}_m$, was PCR amplified from *E. coli* genomic DNA by using primers LDB4 and LDB5. These primers were designed to amplify a 150-bp fragment containing the *metT* gene. A second PCR was performed on this 150-bp fragment by using primers LDB6 and LDB7. The second couple of primers was designed to introduce a T7 RNA polymerase-dependent promoter at the 5′ end of the *metT* gene and an *Mva*I restriction site at the 3′ end. The use of two consecutive PCRs was necessary, since on the *E. coli* chromosome, the *metT* gene is in tandem with the *metU* gene that also encodes $tRNA^{Met}_m$. The final PCR product was cloned into the *Sma*I site of the pUC18 vector, generating the pYL6 plasmid. Transcripts of $tRNA^{Met}_m$ were generated by using T7 RNA polymerase and *Mva*I-digested pYL6 as the template as described previously (17). Full-length transcripts were purified by 10% PAGE.

The purified YggHH6 protein was incubated with AdoMet and [α-^{32}P]GTP-labeled in vitro-transcribed $tRNA^{Met}_m$. After incubation, the tRNA was hydrolyzed using nuclease P1, and the resulting 5′ phosphate nucleotides were analyzed by 2D-TLC and autoradiography. The results showed the formation of m^{7}G in the incubated tRNA (Fig. 2B). To further confirm that m^{7}G formation occurs at position 46, a similar experiment was performed using [α-^{32}P]UTP-labeled $tRNA^{Met}_m$. After incubation in the presence of AdoMet and purified YggHH6, the tRNA was hydrolyzed by RNase T2. The analysis of the resulting 3′ phosphate nucleotides revealed the formation of m^{7}G in the tRNA (Fig. 2B), demonstrating that the m^{7}G produced by YggHH6 is 5′ adjacent to a uridine. In the $tRNA^{Met}_m$, several guanosines are 5′ adjacent to a uridine. To further confirm that

TABLE 2. Bacterial strains and plasmids

Strain or plasmid	Relevant property	Source or reference
E. coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^r)]^c	Stratagene
P4X-SB25	*met* mutant *thr* mutant *relA* Hfr	R. Lavallée
DY330 F′	W3110 Δ*lacU169 gal-490* λcI857 Δ(*cro-bioA*) F′ (*pro-lac*)	D. Bregeon
RDB1	DY330 F′ Δ*yggH*	This study
BL21(DE3)	F$^-$ *ompT* $hsdS_B(r_B^- \ m_B^-)$ *gal dcm* (DE3)	Novagen
Plasmids		
pCR-Blunt II-TOPO	Vector for cloning of PCR fragments	Invitrogen
pCR-*yggH*	*yggH* ORF cloned into pCR-Blunt II-TOPO	This study
pCR-*yggHH6*	*yggHH6* ORF cloned into pCR-Blunt II-TOPO	This study
pET30b	Vector for recombinant protein overexpression	Novagen
pET-*yggHH6*	*yggHH6* ORF cloned into pET30b	This study
pYL6	$tRNA^{Met}_m$ in vitro transcription	This study
pMet(G46A)	$tRNA^{Met}_m$(G46A) in vitro transcription	This study

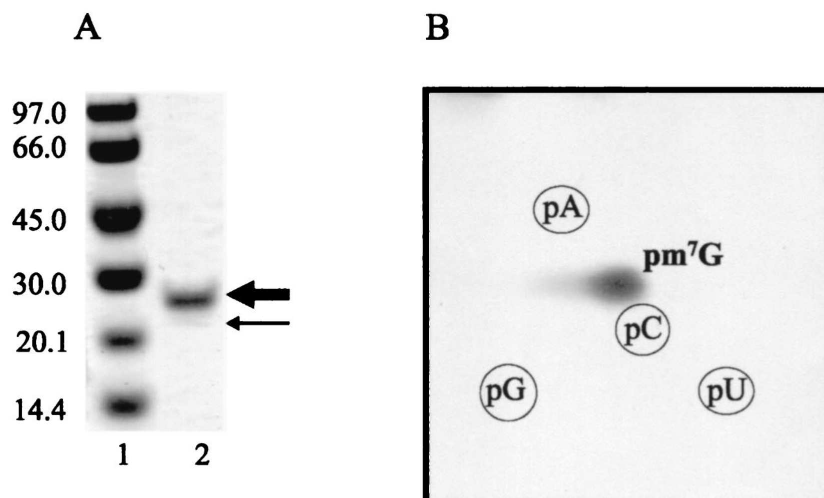

FIG. 1. The product of the *E. coli yggH* ORF catalyzes the formation of m^7G in tRNA. (A) SDS-PAGE of the purified YggHH6 protein. Lane 1, molecular mass markers in kilodaltons (Pharmacia Biotech); lane 2, purified protein. The thick and thin arrows indicate the recombinant YggHH6 protein and its minor contaminant, respectively (see the text for details). (B) Autoradiography of a two-dimensional chromatogram of 5′ phosphate nucleotides on a thin-layer cellulose plate. Total tRNA (100 μg) from the methionine-starved P4X-SB25 strain was incubated in a 200-μl reaction mixture containing 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]-Na (pH 7.0), 4 mM $MgCl_2$, 10 μM [*methyl*-^{14}C]AdoMet (53 mCi/mmol), and 0.4 μg of the purified YggHH6 protein. After a 30-min incubation at 37°C, the tRNA was recovered and digested by nuclease P1, and the resulting nucleotides were analyzed as described previously (12).

m^7G formation occurs at position 46, a mutant $tRNA^{Met}_m$ in which G46 was mutated into A [$tRNA^{Met}_m$(G46A)] was generated by site-directed mutagenesis on the pYL6 plasmid by using primers LDB8 and LDB9. Transcripts of $tRNA^{Met}_m$(G46A) were obtained as described above. The purified YggHH6 protein was incubated with AdoMet and [α-^{32}P]GTP-labeled in vitro-transcribed $tRNA^{Met}_m$(G46A). After incubation, the mutant tRNA was hydrolyzed using nuclease P1, and the resulting 5′ phosphate nucleotides were analyzed by 2D-TLC and autoradiography. The results showed that m^7G was not found in the incubated mutant tRNA (Fig. 2B). Altogether, these results strongly suggest that the purified YggHH6 recombinant protein catalyzes the formation of m^7G at position 46 in *E. coli* tRNA.

The *E. coli yggH* gene is not essential for growth. The *E. coli yggH* gene was inactivated by the insertion of an ampicillin resistance (Ap^r) cassette. This was achieved by homologous recombination, depending on bacteriophage λ recombination functions present in the host strain (18). A linear DNA fragment in which the β-lactamase gene is flanked by 40 bp corresponding to the 5′ and 3′ ends of the *yggH* gene was obtained by PCR using the oligonucleotides LDB10 and LDB11 as primers and plasmid pUC18 as the template. The PCR product was used to transform the DY330 F′(*pro-lac*) strain, and transformants were selected for ampicillin resistance. The presence of the Ap^r cassette in the *yggH* gene in the resulting RDB1 strain was checked by PCR using oligonucleotides LDB12, LDB13, and LDB14 as primers (result not shown). To determine whether m^7G46 formation was affected in the RDB1 strain, crude extracts of the DY330 F′(*pro-lac*) and RDB1 strains were incubated with AdoMet and [α-^{32}P]GTP-labeled in vitro-transcribed $tRNA^{Met}_m$. After incubation, tRNA was hydrolyzed by nuclease P1 and the nucleotides were analyzed by 2D-TLC and autoradiography. The results shown in Fig. 3 revealed the absence of m^7G formation in RDB1 extract. Moreover, when the RDB1 strain was transformed with plasmid pCR-*yggH*, an extract of the resulting strain allowed m^7G formation (Fig. 3). Also, total (crude) tRNA extracted from the wild-type strain DY330 F′(*pro-lac*) was not a substrate for the purified YggH enzyme, while tRNA from the RDB1 strain was an excellent substrate for this enzyme (data not shown). All these data further confirm the role of the YggH protein in the formation of m^7G in tRNA and show that the *yggH* gene is not essential for growth.

Interestingly, a *trmB* mutant (strain GM18) affected in the formation of m^7G in tRNAs was obtained in the 1970s (14). Surprisingly, the *trmB* mutation has been mapped at 6 min and the *yggH* ORF maps at 66 min on the *E. coli* chromosome. Because of this discrepancy, *yggH* cannot yet be renamed *trmB*. A possible explanation for the absence of tRNA (m^7G46) MTase activity in the GM18 strain would be that *trmB* encodes a factor influencing *yggH* expression. Alternatively, two tRNA (m^7G46) MTases might exist in *E. coli*, as has been suggested previously (4). However, the fact that the inactivation of the *yggH* gene leads to a complete absence of tRNA (m^7G46) MTase activity does not support this hypothesis. Further work is required to better characterize the *trmB* mutation.

Sequence analysis of the YggH MTase reveals a distinct family of m^7G MTases. Searches of the sequence database by using PSI-BLAST (2) revealed that orthologs of the *yggH* gene are present in all completely sequenced bacterial genomes and in crown eukaryotes (animals, plants, and fungi), while they are absent from all archaea (data not shown; see also the National Center for Biotechnology Information's COG database at http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG0220). This pattern of phylogenetic distribution is perfectly consistent with the observed presence or absence of m^7G in tRNAs from these organisms (11). Analysis of the multiple sequence alignment (http:

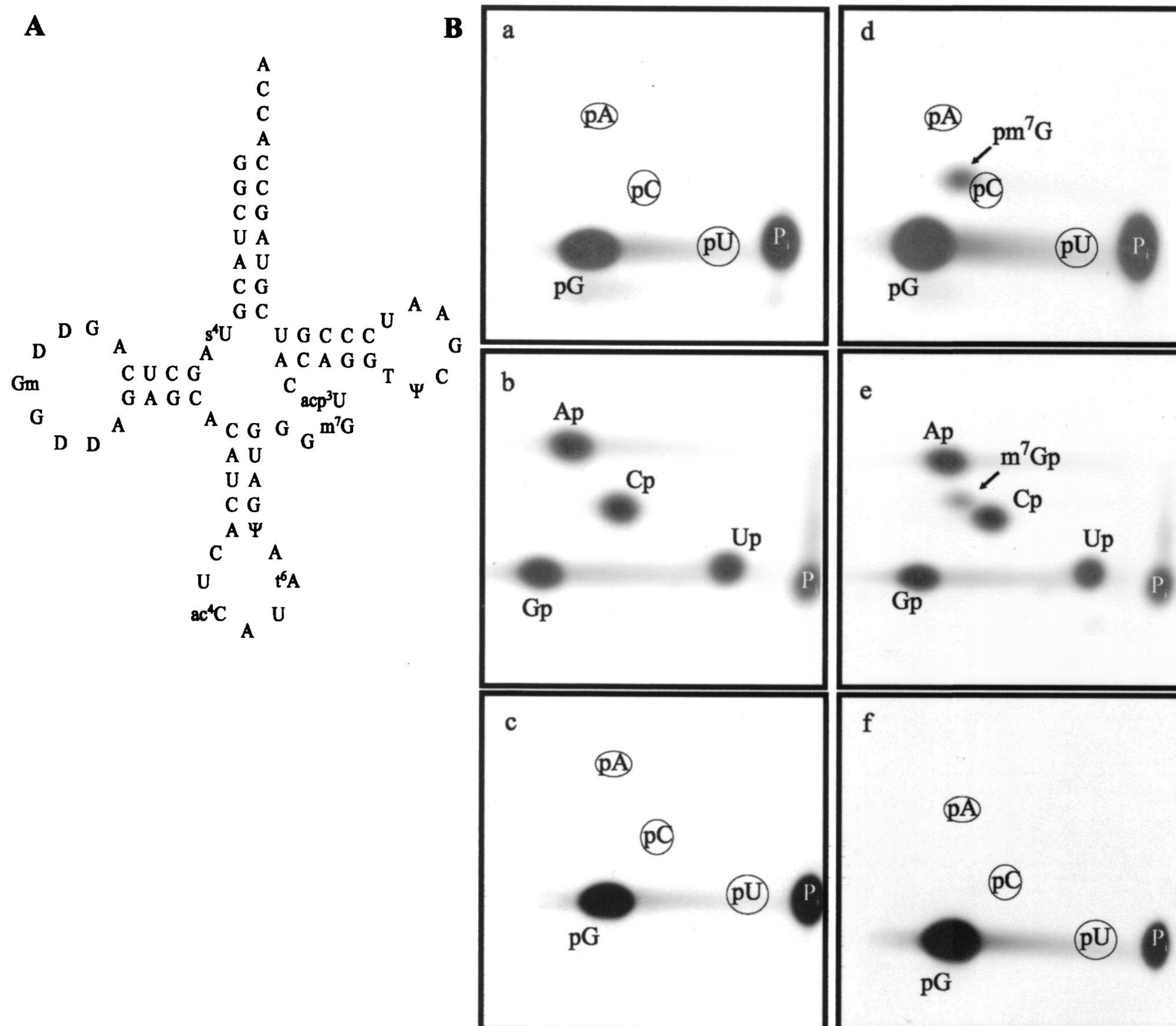

FIG. 2. In vitro-transcribed *E. coli* $tRNA^{Met}_{m}$ is a substrate of the YggH MTase. (A) Cloverleaf representation of the nucleotide sequence of *E. coli* $tRNA^{Met}_{m}$ (9). (B) Autoradiography of two-dimensional chromatograms of 5′ and 3′ phosphate nucleotides on thin-layer cellulose plates. [α-^{32}P]GTP-labeled (a and d) or [α-^{32}P]UTP-labeled (b and e) in vitro-transcribed $tRNA^{Met}_{m}$ and [α-^{32}P]GTP-labeled in vitro-transcribed $tRNA^{Met}_{m}$(G46A) (c and f) (10^6 cpm) were incubated in the presence (d, e, and f) or absence (a, b, and c) of the YggHH6 protein. The reaction mixture contained 50 mM PIPES-Na (pH 7.0), 4 mM $MgCl_2$, 50 μM AdoMet, and 0.4 μg of the purified YggHH6 protein. After 30 min of incubation at 37°C, the tRNA was recovered and digested by nuclease P1 (a, c, d, and f) or RNase T2 (b and e), and the resulting nucleotides were analyzed as described in the legend to Fig. 1.

//www.ncbi.nlm.nih.gov/COG/aln/COG0220.aln) revealed typical MTase motifs in the YggH family and allowed superimposition with the sequences of other m^{7}G MTases acting on different RNAs: the Agr family specific for G1405 within bacterial 16S rRNA (7) and the Abd1 family specific for the cap structure in mRNA (6). The alignment of representative members of the three m^{7}G MTase families (Fig. 4) revealed no striking similarities apart from the residues important for the stability of the common fold or forming the common cofactor-binding pocket. In particular, a tetrapeptide in motif IV, which typically harbors catalytic residues and is very similar in related MTases (10), exhibits completely different patterns of conservation in YggH, Abd1, and Agr, namely, PDPW, CLHY, and PCLE, respectively. It has been argued that the Agr and Abd1 families may use different mechanisms of guanine-N^7 methylation, because the predicted substrate-binding regions and catalytic sites of these enzymes are dissimilar, even though they share a common structural core (7). Identification of the tRNA (m^{7}G46) MTase activity of the *yggH* ORF suggests a third, considerably diverged class of enzymes that generate a similar product (m^{7}G) within a distinct macromolecular context. It remains to be determined whether these three classes of enzymes exhibit any similarities in the m^{7}G methylation mechanism other than the use of a common cofactor and whether they evolved from a common ancestor or independently from various lineages of the MTase superfamily.

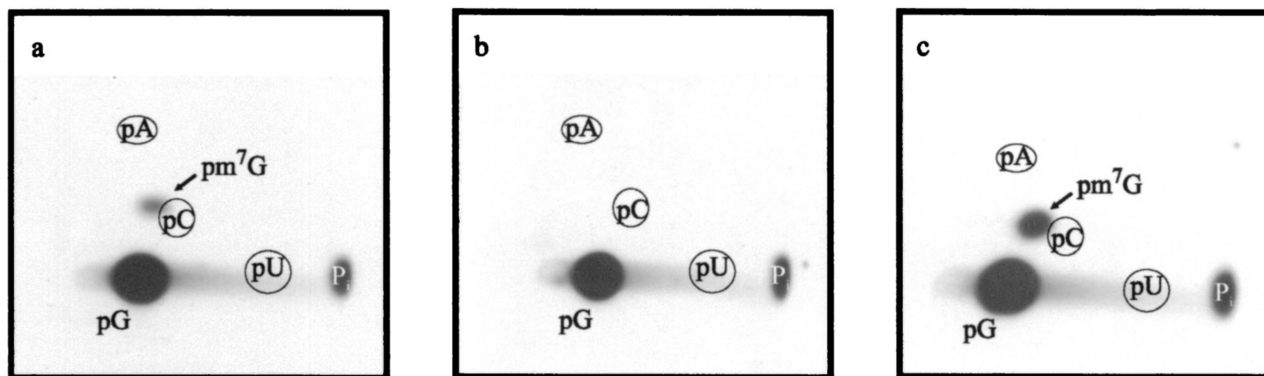

FIG. 3. The *E. coli* RDB1 strain with an inactivated *yggH* gene lacks tRNA (m^7G46) MTase activity. The panels show autoradiography of two-dimensional chromatograms of 5′ phosphate nucleotides on thin-layer cellulose plates. [α-^{32}P]GTP-labeled in vitro-transcribed $tRNA_m^{Met}$ (10^6 cpm) was incubated with a crude extract of the DY330 F′ strain (wild type) (a), of the RDB1 strain (b), or of the RDB1/pCR-*yggH* strain (c). The reaction mixture contained 50 mM PIPES-Na (pH 7.0), 4 mM $MgCl_2$, 50 μM AdoMet, and 100 μg of total protein. After 30 min of incubation at 37°C, the tRNA was recovered and digested by nuclease P1, and the resulting nucleotides were analyzed as described in the legend to Fig. 1.

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ADDENDUM IN PROOF

During the time in which this work was under review, the tRNA (m^7G46) MTase from the yeast *Saccharomyces cerevisiae* was identified (1). Two proteins (Trm8p and Trm82p) forming a complex are required for m^7G46 formation in yeast tRNA. Trm8p appears to be the yeast ortholog of the *E. coli* YggH protein.

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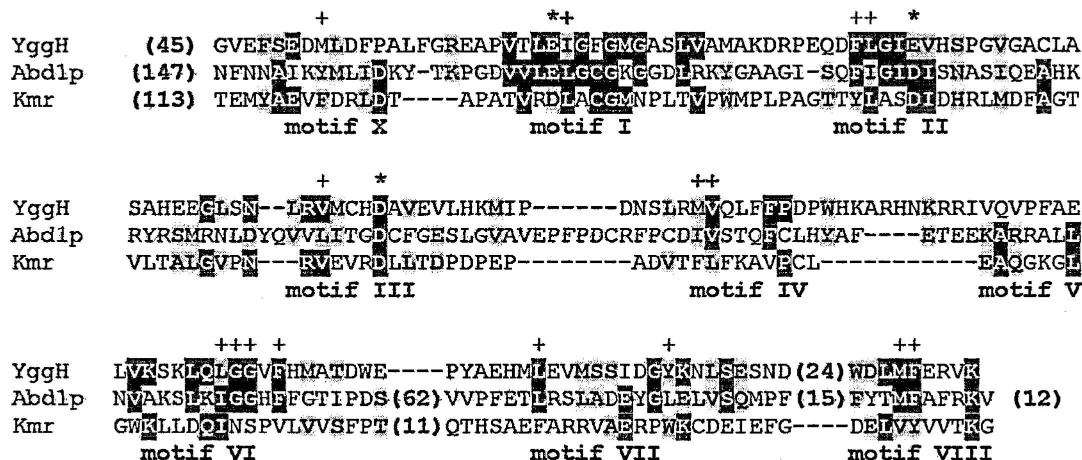

FIG. 4. Sequence alignment of the representative members of three m^7G MTase families specific for tRNA, mRNA, and 16S rRNA: *E. coli* YggH, *S. cerevisiae* Abd1p (cap 0 MTase family), and *Streptomyces kanamyceticus* Kmr (Agr family). Conserved motifs are labeled according to the nomenclature used by Fauman et al. (10). The number of residues omitted for clarity is indicated in parentheses. Conserved AdoMet-binding carboxylate residues are indicated by asterisks, and conserved residues important for the stability of the MTase fold are indicated with pluses.

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