The *Escherichia coli* RlmN methyltransferase is a dual-specificity enzyme that modifies both rRNA and tRNA and controls translational accuracy

ALFONSO BENÍTEZ-PÁEZ,^{1,2} MAGDA VILLARROYA,¹ and M.-EUGENIA ARMENGOD^{1,3,4}

¹Laboratorio de Genética Molecular, Centro de Investigación Príncipe Felipe, 46012 Valencia, Spain

²Bioinformatic Analysis Group—GABi, Centro de Investigación y Desarrollo en Biotecnología, Bogotá D.C., 111221 Colombia

³Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Unidad 721, Centro de Investigación Príncipe Felipe, 46012 Valencia, Spain

ABSTRACT

Modifying RNA enzymes are highly specific for substrate—rRNA or tRNA—and the target position. In *Escherichia coli*, there are very few multisite acting enzymes, and only one rRNA/tRNA dual-specificity enzyme, pseudouridine synthase RluA, has been identified to date. Among the tRNA-modifying enzymes, the methyltransferase responsible for the m²A synthesis at purine 37 in a tRNA set still remains unknown. m²A is also present at position 2503 in the peptidyl transferase center of 23S RNA, where it is introduced by RlmN, a radical S-adenosyl-L-methionine (SAM) enzyme. Here, we show that *E. coli* RlmN is a dual-specificity enzyme that catalyzes methylation of both rRNA and tRNA. The Δ rlmN mutant lacks m²A in both RNA types, whereas the expression of recombinant RlmN from a plasmid introduced into this mutant restores tRNA modification. Moreover, RlmN performs m²A₃₇ synthesis in vitro using a tRNA chimera as a substrate. This chimera has also proved useful to characterize some tRNA identity determinants for RlmN and other tRNA modification enzymes. Our data suggest that RlmN works in a late step during tRNA maturation by recognizing a precise 3D structure of tRNA. RlmN inactivation increases the misreading of a UAG stop codon. Since loss of m²A₃₇ from tRNA is expected to produce a hyperaccurate phenotype, we believe that the error-prone phenotype exhibited by the Δ rlmN mutant is due to loss of m²A from 23S rRNA and, accordingly, that the m²A₂₅₀₃ modification plays a crucial role in the proofreading step occurring at the peptidyl transferase center.

Keywords: RNA modification; anticodon region; peptidyl transferase center; *yfgB*; radical SAM enzymes; translational misreading

INTRODUCTION

Ribosomal and transfer RNAs (rRNAs and tRNAs, respectively) are post-transcriptionally modified in all living organisms. Modification enzymes specifically recognize the target RNA molecule and incorporate the chemical modifications at the precise position. The resulting modified nucleosides can alter RNA chemistry and structure and generally contribute to fine-tune protein synthesis (Grosjean 2005).

tRNA exhibits a larger number and wider variety of modifications than rRNA. In *Escherichia coli* tRNAs, 31 distinct types of modified nucleosides have been identified (Björk and Hagervall 2005), and the set of enzymes

responsible for their synthesis has almost been fully characterized (for brief review, see Benítez-Páez et al. 2010).

The modifications found in the tRNA anticodon loop collectively optimize codon-anticodon recognition, maintain the translational reading frame, and shape the anticodon architecture (Agris 2004, 2008; Björk and Hagervall 2005; Agris et al. 2007). Loss of anticodon modifications, particularly at the wobble position (position 34) and conserved purine 37, frequently impairs gene expression and affects a range of bacterial phenotypic traits, including virulence, pathogenicity, biological fitness, and response to stress conditions (Petrullo et al. 1983; Elseviers et al. 1984; Gray et al. 1992; Li and Björk 1995; Björk et al. 2001; Urbonavicius et al. 2001; Gong et al. 2004; Sha et al. 2004; Cho and Caparon 2008; Golovina et al. 2009; Benítez-Páez et al. 2010). Purine 37 modifications, 3'-adjacent to the anticodon, negate intra-loop base-pairing and, thereby, ensure correct anticodon loop width (Dao et al. 1994). Moreover, they contribute to order the structure of the loop by improving the

⁴Corresponding author

E-mail armengod@cipf.es

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.033266.112>.

stacking of its 3' side and to stabilize the first base pair of the codon-anticodon interaction (Björk and Hagervall 2005; Agris et al. 2007; Agris 2008). In *E. coli* tRNAs, G37 is always modified to m¹G37, whereas A37 may or may not be modified. A weak A-U interaction at the first position of the codon generally requires a complex hydrophobic or charged modification (i⁶A- or t⁶A-type) at position 37, whereas a more stable G-C interaction admits a simpler, hydrophobic modification (m¹G, m²A, m⁶A) or an unmodified A37 (Agris 1996; Björk and Hagervall 2005).

Among the tRNA-modifying enzymes acting at position 37 of the *E. coli* tRNAs, the methyltransferase responsible for m²A synthesis is still unknown. C2-methyladenosine is present at position 37 of tRNA^{Arg}_{ICG}, tRNA^{Asp}_{QUC}, tRNA^{Gln}_{cmnm5s2UUG}, tRNA^{Gln}_{CUG}, tRNA^{Glu}_{mm5s2UUG}, and tRNA^{His}_{QUG} from *E. coli* (Czerwoniec et al. 2009). Identification of the gene encoding this tRNA modifying enzyme could shed light on the role of m²A₃₇ in translation and bacterial physiology.

The m²A nucleoside is also present at A2503 in 23S rRNA, where it is introduced by RlmN. m²A2503 is located at the entrance of the nascent peptide exit tunnel and has been involved in the ribosomal mechanism that promotes translation arrest in response to specific peptide sequences (Vazquez-Laslop et al. 2010; Ramu et al. 2011). Lack of methylation of A2503 has been reported to produce a subtle yet significant effect on cell fitness and to increase linezolid resistance (Toh et al. 2008; Gao et al. 2010; Lamarre et al. 2011). RlmN and its evolutionarily related resistance enzyme Cfr belong to the radical S-adenosyl-L-methionine (SAM) enzyme superfamily, and both use protein-free rRNA as a substrate (Atta et al. 2010; Kaminska et al. 2010; Yan et al. 2010; Grove et al. 2011; Yan and Fujimori 2011).

Modifying RNA enzymes are highly specific for substrate (RNA type) and the target position. In *E. coli*, there are very few multisite acting enzymes (RluD, RsmA, RlmKL, TruA, and DusABC), and only one enzyme, the pseudouridine synthase RluA, has been shown to have dual specificity, recognizing both rRNA and tRNA (Fig. 1). In this work, we demonstrate that *E. coli* RlmN catalyzes the incorporation of m²A at position 37 of tRNAs. Thus, RlmN is the first example of a dual-specificity RNA methylase which catalyzes m²A synthesis in both rRNA and tRNA. Moreover, we show that inactivation of the *rlmN* gene results in an error-prone phenotype, which highlights a role for nucleoside m²A in translational accuracy.

RESULTS AND DISCUSSION

RlmN catalyzes m²A formation in tRNA

Synthesis of m²A involves the addition of a methyl group to an amidine carbon of the adenine, which is a highly chemically challenging reaction because of the low nucleophilicity of the target carbon atom. RlmN and its closely related

homolog Cfr catalyze this reaction on 23S rRNA (Toh et al. 2008; Giessing et al. 2009; Yan et al. 2010; Grove et al. 2011; Yan and Fujimori 2011). Therefore, as a first attempt to identify the enzyme responsible for synthesizing m²A in tRNAs, we performed a search for RlmN/Cfr paralogs in databases. However, BLAST searches of the *E. coli* and other bacterial genomes using conserved amino acid blocks of the RlmN family as queries revealed no paralog that might function in tRNA modification, which is in agreement with previous studies (Toh et al. 2008; Kaminska et al. 2010).

Then, we tested the hypothesis that RlmN is also responsible for the modification of tRNAs. This hypothesis was based on the observation that m²A-carrying tRNAs share certain sequence similarity with 23S rRNA in the region including the target nucleoside (Fig. 2A). Moreover, the global L-shaped structure of tRNAs resembles the local structure of 23S rRNA required for recognition by RlmN (helices 89 and 90–92) (Fig. 2B). Fractions of tRNA and rRNA (23S rRNA-enriched) purified from Δ *rlmN* and wild-type strains were analyzed by high-performance liquid chromatography (HPLC) after digestion with nuclease P1 and alkaline phosphatase. Nucleoside m²A, which elutes at ~54.5 min, was found to be absent in the hydrolysates of rRNA and tRNA purified from the Δ *rlmN* mutant (Fig. 3A). To test whether the presence of m²A in tRNA is truly dependent on RlmN activity, the sequence encoding the full-length RlmN protein with an N-terminal histidine tag was cloned into pET15b, and the resulting plasmid was used to transform the Δ *rlmN* mutant. The recombinant plasmid was able to restore the presence of m²A in bulk tRNA (Fig. 3B). Note that the in vivo modification reaction proceeded efficiently in a host strain lacking the T7 RNA polymerase gene. Low-level synthesis of the His-RlmN recombinant protein could be due to transcription mediated by bacterial RNA polymerase from the T7 promoter (non-specific transcription) and/or other plasmid promoters. Under such conditions, the His-RlmN expression was so low that it could not be detected by Western blotting with an anti-His antibody (data not shown). In spite of this, incorporation of m²A into tRNA was indicative of the RlmN expression from the plasmid-borne *rlmN* gene.

Construction and characterization of tRNA^{Chimera}_{UUG}

To further investigate the effect of RlmN activity on tRNA, we planned to express tRNA^{Gln}_{cmnm5s2UUG} from a tRNA scaffold system (Ponchon and Dardel 2007; Ponchon et al. 2009), which could facilitate the production, purification, and mutational analysis of the recombinant tRNA. This approach has been successfully used by our group in a previous study involving the tRNA^{Leu}_{CmAA} isoacceptor (Benítez-Pérez et al. 2010). However, no accumulation of several chimeric constructs involving tRNA^{Gln}_{cmnm5s2UUG} and other tRNA substrates was herein detected, probably because they are unstable and undergo degradation by

Modifications of the *E. coli* tRNA and rRNA

FIGURE 1. RlmN is the first example of a methyltransferase working on both rRNA and tRNA. The figure shows the distribution of the *Escherichia coli* modified nucleotides by RNA type. The position of each modification (in parentheses) and the enzyme(s) responsible for its synthesis are indicated. Only two enzymes, RluA and the herein studied RlmN, have dual substrate specificity, recognizing both rRNA and tRNA. (*) Modifications of 16S rRNA; (unk) unknown enzyme; (‡) modification h⁵C2501 is partial and dependent on the growth phase (Andersen et al. 2004; Havelund et al. 2011).

endogenous RNases (see Materials and Methods). In contrast, we observed the overexpression of tRNA^{Chimera}_{UUG}, which was constructed by replacing the anticodon stem-loop (ASL) of the tRNA scaffold with the ASL of tRNA^{Gln}_{cmnm5s2UUG} (Fig. 4). The HPLC profile of tRNA^{Chimera}_{UUG} indicated the presence of the normally occurring modifications in the tRNA scaffold (such as s⁴U₈, m⁷G₄₆, T₅₄, D_{16/20}, and Ψ₅₅) (see Fig. 4A) when overexpressed in *E. coli* (Ponchon and Dardel 2007). Additionally, we identified the five modifications typically present in the tRNA^{Gln}_{cmnm5s2UUG} ASL (Czerwonec et al. 2009), with Um at position 32, s²U and cmnm⁵U at position 34, m²A at position 37, and Ψ at position 38 (whose presence was inferred from the increase of the Ψ peak area in the chromatogram) (Fig. 4B). These data suggest that tRNA^{Chimera}_{UUG} may be an appropriate model to investigate RlmN activity. In fact, an A37C replacement in the chimeric tRNA, and the overproduction of the wild-type tRNA^{Chimera}_{UUG} in Δ rlmN cells allowed us to demonstrate that the m²A modification is confined to position 37 and that it is dependent on RlmN activity, respectively (Fig. 4C).

In vitro methylation of tRNA by RlmN

Purification of the enzymatically competent RlmN protein demands strict anaerobic conditions to allow the proper assembly of its Fe-S cluster (Yan et al. 2010). An in vitro reconstitution of an Fe-S-bound RlmN protein was herein

performed by the degassing and N₂ purging of buffers, reagents, and solutions as an alternative method to using a glovebox device (Yan et al. 2010). N₂ purging efficiently removes O₂ from aqueous solutions, leaving oxygen levels at 0.2–0.4 ppm (Butler et al. 1994). The recombinant His-RlmN protein purified by this procedure was able to catalyze the synthesis of m²A in a SAM-dependent manner on tRNA^{Chimera}_{UUG} purified from Δ rlmN cells (Fig. 4D). In vitro accumulation of m²A was calculated to be ~10%–20% of that observed in vivo by comparing the peak areas of m²A (normalized to the cytidine area) from the HPLC profiles of the in vitro (Fig. 4D) and in vivo (Fig. 4C) modified tRNA. Interestingly, when we attempted to assay RlmN activity using in vitro-transcribed tRNA^{Gln}_{UUG} or tRNA^{Arg}_{ACG}, no trace of m²A was observed in the HPLC analysis of the reaction products (Fig. 4D; data not shown). These results suggest that unmodified tRNA is not a suitable substrate for RlmN, as also observed for other tRNA modifying enzymes (Benítez-Páez et al. 2010).

tRNA determinants for RlmN and other tRNA-modifying enzymes

The ability of RlmN to modify rRNA and tRNA supports our initial hypothesis that both RNAs have common identity determinants for the enzyme (see Fig. 2). At the sequence level, positions 35 and 36 in the tRNAs recognized by RlmN show a clear pattern of nucleotide conservation

FIGURE 2. Sequence and structural comparisons of the RlmN substrates. (A) Sequence and secondary structure of the peptidyl transferase region of the *E. coli* 23S rRNA. Modification m^2A at position 2503 is highlighted in black. The remaining post-transcriptional modifications in the region are highlighted in gray. Note location of helices 90–92, which are crucial for recognition by RlmN. In the *bottom right* box, a short multiple sequence alignment (seven positions in length) of 23S rRNA and the tRNAs carrying m^2A_{37} is used to search for the potential identity determinants recognized by the m^2A -synthesizing enzyme. Positions at the consensus pattern are represented according to the IUPAC nucleotide code. Positions 35 and 36 of substrate tRNAs showing a significant conservation pattern were chosen herein for subsequent studies. (B) The three-dimensional structure corresponding to 2455–2580 nucleotides of the *Escherichia coli* 23S rRNA sequence was extracted from the 30AS structure stored in the PDB database and shown in ribbon representation (*left-hand side*) using UCSF Chimera viewer (Pettersen et al. 2004). Helices required for optimal RlmN action (Yan et al. 2010) are depicted in color, and the target nucleoside for the RlmN-mediated methylation is highlighted in red. The three-dimensional, global structure of the *Escherichia coli* tRNA^{Gln}_{CUG} (extracted from the 2RE8 PDB record) is shown in ribbon representation on the *right-hand side*. The D, anticodon, and T stem-loops are depicted in color, resembling the secondary structures shown on the *left-hand side*. The target nucleoside for RlmN-mediated modification is also highlighted in red.

(G or C at position 36 and a pyrimidine at position 35), as also observed at the counterpart positions in 23S rRNA (Fig. 2A). Therefore, we performed site-directed mutagenesis on the plasmid containing tRNA^{Chimera}_{UUG} to produce the U35A, U35G, G36U, and G36A changes and to analyze their effect on m^2A synthesis. All the tRNA mutants were overexpressed at similar or slightly lower levels than tRNA^{Chimera}_{UUG} (Supplemental Fig. S1). The results of HPLC analysis are summarized in Table 1. We found that the accumulation of m^2A in the mutants was ~20%–50% lower than in the wild-type tRNA^{Chimera}_{UUG}, which suggests that U35 and G36 work as identity determinants for RlmN.

Post-transcriptional modifications at position 37 may change to match a mutational change in the nucleotide at position 36 (Yarus 1982; Raftery and Yarus 1987). However, the decrease in m^2A produced by the mutations at G36 in tRNA^{Chimera}_{UUG} was not accompanied by an accumulation of alternative adenosine nucleosides at position 37. Specifically, we observed neither t^6A accumulation in mutant G36U (whose anticodon 5'-UUU is characteristic of tRNA^{Lys}_{mm5s2UUU}) nor $(ms^2)i^6A$ accumulation in mutant G36A (whose anticodon 5'-UUA may recognize the stop codons 5'-UAA and 5'-UAG). The G36A mutant is a putative substrate for MiaA, the enzyme that introduces i^6A_{37} into tRNA reading codons starting with U, as a first step in the synthesis of the final modification $ms^2i^6A_{37}$. MiaA predominantly recognizes a conserved consensus sequence $A_{36}A_{37}A_{38}$ in the anticodon loop of tRNA (Chimnaronk et al. 2009). However, some tRNA chimeras carrying a sequence $A_{36}A_{37}\Psi_{38}$ have been shown to carry both $ms^2i^6A_{37}$ and m^2A_{37} (Yarus et al. 1986). Apparently, this is not the case for our mutant chimera G36A which, even though containing a sequence $A_{36}A_{37}\Psi_{38}$, is not recognized by MiaA. Modification t^6A_{37} , found in ANN decoding tRNAs (such as tRNA^{Lys}_{mm5s2UUU}), is dependent on activity of RimN (previously named YrdC), although the identity elements recognized by this enzyme

FIGURE 3. RlmN modifies both rRNA and tRNA. (A) rRNA/tRNA HPLC-nucleoside profile of the *Escherichia coli* $\Delta rlmN$ mutant. After isolation and P1 digestion, bulk rRNA (left) and tRNA (right) from the wild-type and $\Delta rlmN$ strains were separately analyzed by HPLC. Nucleoside m²A was identified by its elution time and UV spectra. Note that nucleoside t⁶A (present at position 37 of some tRNAs that are not RlmN substrates) is unaffected in the $\Delta rlmN$ cells. (B) In vivo complementation of the $\Delta rlmN$ mutant. Recombinant protein His-RlmN, expressed from pET15b-his-rlmN, restores the m²A synthesis in $\Delta rlmN$ cells (right panel). Wild-type and $\Delta rlmN$ cells containing pET15b (left and middle panels, respectively) were used as controls. Absorbance was monitored at 260 nm. (mAU) Absorbance units $\times 10^{-3}$.

have not yet been determined (El Yacoubi et al. 2009). Lack of t⁶A₃₇ in mutant G36U indicates that the generation of an anticodon that properly matches an ANN codon does not suffice for recognition by RimN.

Additionally, we observed that mutation of U35 and G36 affects modifications at positions of tRNA^{Chimera}_{UUG} other than position 37. Thus, mutations G36A and G36U diminish the accumulation of Ψ and s⁴U to a certain extent, while mutation G36A also lowers the s²U level to $\sim 50\%$ (Table 1). MnmA catalyzes s²U₃₄ formation in tRNA species with anticodons 5'-UUG (glutamine), 5'-UUU (lysine), and 5'-UUC (glutamate) by recognizing U34 and U35 in a base-specific manner (Numata et al. 2006). Our results suggest that the presence of an A at position 36 (instead of G, U, or C) hinders the MnmA function. As expected, mutations U35A and U35G drastically impaired the synthesis of s²U₃₄ by MnmA in the chimeric tRNA and, consequently, accumulation of the cmnm⁵s²U nucleoside (Table 1). Strikingly, both mutations induced the incorporation of the cmo⁵ group at the wobble uridine. This

modification is present in tRNAs that read codons in family boxes specific for Leu, Ser, Pro, Val, Thr, and Ala (Nasvall et al. 2004; Björk and Hagervall 2005; Nasvall et al. 2007). Formation of cmo⁵U₃₄ in mutants U35A (with anticodon 5'-UAG) and U35G (with anticodon 5'-UGG) is consistent with the modification status at the wobble uridine in tRNA^{Leu}_{cmo5UAG} and tRNA^{Pro}_{cmo5UGG}. Therefore, an A or G nucleotide at position 35 of the composite tRNA appears as a critical identity determinant for its recognition by the cmo⁵U₃₄ synthesizing enzymes.

A further comparison of the 23S rRNA and tRNA^{Chimera}_{UUG} sequences revealed some additional similarities which could be used for recognition by RlmN. A Ψ residue is located 3'-adjacent to the RlmN-target nucleotide in both RNAs, and a 2'-O-methylation is present in the pyrimidine separated by four nucleotides toward the 5' end from the RlmN target (Figs. 2A, 4B). Thus, we decided to explore the effect of inactivating genes *truA* and *trmJ* (responsible for pseudouridilation at position 38 and 2'-O-methylation at U32, respectively) on m²A accumulation in tRNA^{Chimera}_{UUG}. No significant differences were found between the wild-type strain and mutants $\Delta truA$ and $\Delta trmJ$ (Table 1).

In order to investigate the role of the tRNA structure in the recognition by RlmN, we attempted to clone truncated

versions of the tRNA^{Chimera}_{UUG} lacking either the D stem-loop or the T stem-loop. Unfortunately, no overexpression of any truncated mutant was obtained.

Inactivation of *rlmN* decreases translational accuracy

Nucleoside m²A is located in functionally important regions of tRNA and rRNA. However, there are no data available on a role of m²A in translational accuracy. To test the fidelity phenotype of the $\Delta rlmN$ mutant, we used a nonsense readthrough assay in which decoding of a *lacZ* premature stop codon by near-cognate tRNAs is required to produce β -galactosidase activity. This assay has previously proved useful to characterize the effect of several tRNA modification enzymes (Petrullo et al. 1983; Elseviers et al. 1984; Yim et al. 2003). As shown in Table 2, mutations $\Delta miaA$ (causing the loss of ms²i⁶A₃₇ in a tRNA set), and $\Delta mnmE$ and $\Delta mnmA$ (both impairing wobble uridine modifications in certain tRNAs), used here as controls, produce an error-restrictive phenotype, i.e., they lower the

FIGURE 4. RlmN methylates tRNA^{Chimera}_{UUG} both in vivo and in vitro. (A) The sequence and secondary structure of the scaffold tRNA is shown together with a representative section of its HPLC chromatogram (D and Ψ modifications elute at early times and are not included in the figure). (B) The tRNA^{Chimera}_{UUG} sequence (red nucleotides indicate the ASL from tRNA^{Gln}_{cmnm5s2UUG}) is shown together with a section of the corresponding chromatogram. (C) Synthesis in vivo of m²A on tRNA^{Chimera}_{UUG} (left panel) is prevented by mutation A37C (middle panel) or by the expression of the wild-type tRNA^{Chimera}_{UUG} in Δ rlmN cells (right panel). (D) In vitro methylation of tRNA^{Chimera}_{UUG} purified from Δ rlmN cells is carried out by in vitro-reconstituted RlmN in a SAM-dependent manner (red lines). Reconstituted RlmN does not work on in vitro synthesized tRNA^{Gln}_{UUG} (black lines). Solid and discontinuous lines indicate that the modification reactions were performed in the presence and absence of SAM, respectively. Absorbance was monitored at 260 nm. (mAU) Absorbance units $\times 10^{-3}$.

readthrough level in relation to the wild-type strain. These results suggest that tRNAs that are substrates for MiaA, MnmE, and MnmA misread the *lacZ* UAG codon, recognizing it as a near-cognate codon and contributing to the natural low-level readthrough detected in wild-type cells. Presumably, loss of the MiaA-, MnmE-, and MnmA-dependent modifications destabilizes the weak pairing of tRNAs with the stop codon at the decoding center, thus reducing the readthrough level.

The readthrough assay has also proved useful to assess the effects of changes in ribosomal components on translational accuracy (Petrullo et al. 1983). In our hands (see Table 2), the assay successfully characterized the well-known error-prone and error-restrictive phenotype conferred by mutations *rpsD12* (in ribosomal protein S4) and *rpsLK42T* (in ribosomal protein S12), respectively (Petrullo et al. 1983; Kurland et al. 1996). Mutations in proteins S4 and S12 affect the conformational changes occurring at the decoding center during the tRNA selection step (Vallabhaneni and Farabaugh 2009), thus stimulating the selection or rejection of near-cognate tRNAs.

Interestingly, *rlmN* inactivation produces a clear error-prone phenotype since it increases the UAG readthrough levels in relation to the wild-type strain (Table 2). This effect is opposite to that observed in Δ *miaA*, where loss of modification at position 37 decreases the UAG readthrough. All the tRNAs that are substrates for RlmN carry G or C at position 36, which involves a noncanonical interaction with the first base of the UAG stop codon. Considering that most purine 37 modifications are required for stabilization of the crucial first base pair of the anticodon–codon interaction, it appears unlikely that the error-prone phenotype of the Δ *rlmN* mutant is due to loss of m²A from tRNAs because this would mean that m²A-lacking tRNAs form more stable interactions with the *lacZ* stop codon than fully modified tRNAs.

Alternatively, we propose that the error-prone phenotype exhibited by the Δ *rlmN* mutant is due to loss of m²A from ribosomal 23S. Nucleotide m²A2503 of 23S RNA resides in the peptidyl transferase center (PTC). Besides its crucial role in peptide-bond formation, this center is involved in the discrimination of near-cognate tRNAs during

TABLE 1. Mutations affecting the modification status of tRNA^{Chimera}_{UUG}

	tRNA ^{Chimera} _{UUG} mutants purified from the wild-type strain				tRNA ^{Chimera} _{UUG} purified from the mutant strain	
	A37C	U35A	U35G	G36U	G36A	$\Delta trmJ$ $\Delta truA$
ASL modifications	Um32	1.32 ± 0.33	1.09 ± 0.03	1.22 ± 0.13	1.07 ± 0.02	0.98 ± 0.07
	cmnm ⁵ s ² U34	1.68 ± 0.42	0.00	0.00	0.31 ± 0.11	0.74 ± 0.03
	cmo ⁵ U34	0.00	1.03 ± 0.01	0.98 ± 0.05	0.00	0.00
	s ² U34	1.04 ± 0.07	0.00	0.00	0.94 ± 0.01	0.54 ± 0.08
	m ² A37	0.00	0.72 ± 0.12	0.81 ± 0.15	0.54 ± 0.06	0.70 ± 0.19
Core modifications	Ψ38 (+Ψ55)	0.88 ± 0.04	1.21 ± 0.01	1.05 ± 0.01	0.69 ± 0.01	0.69 ± 0.02
	s ⁴ U8	0.90 ± 0.32	1.05 ± 0.02	1.43 ± 0.25	0.89 ± 0.01	0.83 ± 0.15
	D16, D20	1.32 ± 0.28	1.09 ± 0.03	1.22 ± 0.13	1.07 ± 0.02	0.98 ± 0.07
	m ⁷ G46	0.91 ± 0.02	0.91 ± 0.01	0.92 ± 0.03	0.88 ± 0.01	0.85 ± 0.01
	m ⁵ U54	0.94 ± 0.02	1.00 ± 0.01	0.99 ± 0.08	1.00 ± 0.01	0.96 ± 0.03

Nucleoside quantitation was calculated from the peak areas in the HPLC chromatograms as follows: peak area of nucleoside X (normalized to the cytidine area) in the mutant (tRNA or strain)/peak area of nucleoside X (normalized to the cytidine area) in the wild-type tRNA purified from the wild-type strain. Nucleosides were monitored at 260 nm. In the case of cmo⁵U, numbers were calculated by considering the average accumulation of cmo⁵U in mutants U35A and U35G as the reference value (denominator) since this nucleoside is not present in the wild-type tRNA^{Chimera}_{UUG}. Quantitative analysis of nucleoside accumulation is presented as the mean of two independent experiments ± SEM. Diminished accumulation of nucleoside Ψ is thought to result from the impaired modification at position 38; therefore, Ψ values ~0.50 (see the $\Delta truA$ mutant) are assumed as 0% of Ψ38 accumulation.

TABLE 2. *rlmN* inactivation confers an error-prone phenotype

Strain ^a	β-galactosidase activity ^b
Wild-type	5.20 ± 0.70
<i>ΔmiaA</i>	0.73 ± 0.08**
<i>ΔmnmE</i>	1.00 ± 0.30*
<i>ΔmnmA</i>	0.53 ± 0.11**
<i>rpsD12</i>	15.60 ± 0.40***
<i>rpsL42T</i>	0.02 ± 0.01***
<i>ΔrlmN</i>	12.40 ± 1.30**

^aAll the strains were derivatives of IC4639 which contains a UAG stop codon in the *lacZ* gene.

^bReadthrough of the *lacZ* UAG codon was determined as indicated in the Materials and Methods. β-galactosidase activity is expressed as Miller units (Miller 1990). Each value is the mean ± SEM of at least four independent experiments. Data were compared for statistical significance using a *t*-test with Welch's correction. (*) $P \leq 0.012$, (**) $P \leq 0.009$, (***) $P \leq 0.001$.

the proofreading step (Jenner et al. 2010). The location of A2503 suggests that loss of m²A may alter conformational changes in the PTC, allowing the accommodation of an incorrect A-site tRNA and the subsequent peptidyl-transfer catalysis, thus increasing the readthrough levels. If this were the case, it would indicate that the rRNA modification catalyzed by RlmN serves to optimize ribosomal fidelity.

Concluding remarks

We show herein that RlmN is the methyltransferase responsible for m²A synthesis at position 37 in a group of *E. coli* tRNAs. RlmN activity on tRNA was demonstrated in vivo by the absence of m²A in total tRNA purified from a *ΔrlmN* mutant (Fig. 3A) and by restoration of tRNA methylation upon the expression of a plasmid-encoded *rlmN* gene in the same strain (Fig. 3B). In vivo RlmN activity on tRNA was confirmed by the presence of m²A in a chimeric tRNA (tRNA^{Chimera}_{UUG}) purified from the wild-type strain and by the absence of the modification when this tRNA was purified from the *ΔrlmN* mutant (Fig. 4C, left and right panels). Moreover, the A37C replacement in tRNA^{Chimera}_{UUG} prevented the RlmN-dependent formation of m²A, indicating that this nucleoside is confined to position 37 (Fig. 4C, left and middle panels). We also demonstrate that RlmN, after in vitro reconstitution of its iron-sulfur cluster, carries out the synthesis in vitro of m²A on tRNA^{Chimera}_{UUG} purified from *ΔrlmN* cells (Fig. 4D). Therefore, our data evidence that RlmN is a dual-specificity enzyme capable of catalyzing m²A formation in both rRNA and tRNA. This is the first example of a methyltransferase that works on both types of RNAs (Fig. 1).

The tRNA chimera herein constructed also proves useful to study the tRNA identity determinants for RlmN (Table 1). By using site-directed mutagenesis on the plasmid containing tRNA^{Chimera}_{UUG}, we produced the U35A, U35G, G36U, and G36A variants. We found that change G36U, as well as the

other variants albeit to a lesser extent, reduce m²A accumulation, suggesting that nucleosides at positions 35 and 36 of the anticodon help the recognition of tRNA by RlmN.

RlmN in vitro modifies tRNA^{Chimera}_{UUG} purified from *ΔrlmN* cells but not two unmodified substrates (tRNA^{Gln}_{UUG} and tRNA^{Arg}_{ACG}) obtained by in vitro transcription (Fig. 4D; data not shown). This suggests that RlmN works in a late step during tRNA maturation. No modification in the ASL region of tRNA^{Chimera}_{UUG} seems crucial for m²A synthesis (Table 1), but modifications in other tRNA regions contributing to stabilizing the L-shaped tertiary structure may be required for RlmN action. RlmN activity on 23S rRNA is dependent on the presence of helices 90–92 (Yan et al. 2010). Likewise, RlmN may require a precise 3D structure of tRNA to perform m²A synthesis at position 37. Unfortunately, we were unable to obtain stable tRNA^{Chimera}_{UUG} derivatives lacking the D or the T stem-loop. Construction of new, stable derivatives of tRNA substrates for RlmN or the determination of crystal structures of RlmN in the apo- and tRNA-bound forms might help characterize the tRNA identity elements for RlmN and discover what they have in common with those in 23S rRNA.

The overexpression in a wild-type strain of tRNA^{Chimera}_{UUG} and its mutants also provides valuable information about the identity determinants used by certain tRNA modifying enzymes, such as RimN and CmoA/CmoB, whose tRNA recognition mechanisms remain to be investigated (Table 1).

RlmN inactivation produces an error-prone phenotype since it increases the misreading of a UAG stop codon (Table 2). This phenotype is opposite to the error-restrictive phenotype expected for the mutations removing modifications at position 37 and exemplified by *ΔmiaA* (Table 2). Therefore, we suspect that the misreading effect of *ΔrlmN* is due to the lack of m²A in the rRNA, which would facilitate the accommodation of an incorrect tRNA in the PTC. In the readthrough assay herein used, the error-prone effect produced by loss of m²A from the PTC could compensate for the error-restrictive effect due to the absence of m²A in tRNAs. Certainly, the simultaneous location of m²A in tRNA and rRNA complicates the study of its precise role in translational accuracy. The construction of in vivo and in vitro systems allowing a combination of fully modified tRNAs with m²A-lacking ribosomes, or vice versa, would facilitate the analysis of the individual effect of m²A on rRNA or tRNA functioning, respectively.

Modifications in rRNAs cluster in ribosomal regions of functional importance and are thought to collectively contribute to the fine-tuning of the translation process (Decatur and Fournier 2002; Chow et al. 2007). However, there is so far no known role for these modifications in ribosomal accuracy, except for dimethylation introduced by KsgA (RsmA) into A1518 and A1519 of 16S rRNA, and the N⁴ and 2'-O-methylations introduced by RsmH and

RsmI proteins at position 1402 of 16S rRNA (van Buul et al. 1984; Kimura and Suzuki 2010). If, as we believe, the error-prone phenotype of the $\Delta rlmN$ mutant is due to the lack of m²A in 23S rRNA, then our data would represent the first example of how a single 23S RNA modification at the PTC plays a crucial role in ribosomal accuracy.

MATERIALS AND METHODS

Sequence analysis and candidate selection

Using the RlmN sequence from *E. coli* (Uniprot id P36979) as a query, a Blastp search against the nonredundant Reference Sequences Database at NCBI (Pruitt et al. 2012) was conducted with default parameters (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al. 1997). We manually explored the phylogenetic distribution of RlmN homologs in major Eubacteria phyla (i.e., Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Chlamydiae, Cyanobacteria, Deinococci, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes, and Thermotogae). Based on pairwise comparisons with alignment coverage >75% and alignment score >60 bits, we retrieved more than 130 different sequences that are representative of diversity for each bacterial phylum. The full set of RlmN homologs was aligned using the Probcons software, v1.12, with 1000 passes of iterative refinement (Do et al. 2005) followed by a filtering for gaps. An RlmN amino acid profile was constructed using the HMMER v2.3.2 algorithm and its default parameters (Eddy 1998). Then, search against nonredundant nucleotide and protein databases from *Escherichia coli* K-12 was carried out with the TBLASTN and Blastp algorithms, respectively, using the different conserved blocks of amino acids obtained from HMMER as queries. Afterward, no paralogs of RlmN were found. We extended this search to other model bacterial genomes and obtained similar results.

Bacterial strains, plasmids, oligonucleotides, and DNA manipulations

E. coli strains and plasmids used in this study are listed in Table 3. Oligonucleotides and primers are described in Supplemental Table S1. P1 transductions were performed following standard procedures (Miller 1990). Kanamycin-resistant transductants were verified by PCR using a primer flanking the gene replaced with the *kan* cassette and an internal primer for *kan*, as recommended (Datsenko and Wanner 2000). The transductants carrying the *rpsLK42T* or *rpsD12* allele were verified by PCR amplification and sequencing. DNA manipulations were carried out according to standard protocols. Point mutations were obtained by site-directed mutagenesis with appropriate PCR primers. The resulting constructs were verified by DNA sequencing.

Isolation of bulk rRNA/tRNA and analysis of the RNA modification status by HPLC

Bacterial strains were grown overnight in LB medium, 1/100 diluted in 100 mL of the same medium and then grown to an OD₆₀₀ of 0.7–0.8. Cells were harvested by centrifugation and resuspended in 0.4 mL of buffer-A (25 mM Tris pH 7.4, 60 mM KCl, 10 mM MgCl₂). Lysozyme (2 mg, Sigma) was added, and the

suspension was incubated at 37°C for 15–20 min. The cell suspension was lysed by three freeze-thaw cycles using liquid nitrogen; then 0.6 mL of buffer-B (buffer-A supplied with 0.6% Brij35, 0.2% Na-deoxycholate, 0.02% SDS) and 0.1 mL of phenol (equilibrated to pH 4.3 with citrate) were added and mixed. The suspension was incubated on ice for 15 min, and the aqueous phase was extracted twice with 1 vol of phenol. RNA was precipitated with 2.5 vol of cold ethanol containing 1% (w/v) potassium acetate. The pellet was washed with 70% ethanol and dissolved in 2 mL of buffer R200 (100 mM Tris-H₃PO₄, pH 6.3, 15% ethanol, 200 mM KCl) prior to running over a Nucleobond AX500 column (Macherey-Nagel), pre-equilibrated with 10 mL of the same buffer. The column was washed once with 6 mL of R200 and once with 2 mL of R650 (R200 with 650 mM KCl). tRNA was eluted with 6 mL of R650 buffer and precipitated with 0.7 vol of isopropanol, washed with 70% ethanol and redissolved in water. After tRNA elution, columns were washed with 6 mL of R1200 (R200 with 1.2 M KCl), which led to elution of 16S-enriched rRNA, and subsequently with 6 mL R1500 (R200 with 1.5 M KCl), which permitted us to obtain a 23S-enriched rRNA fraction. Given that the m²A modification resides only in 23S rRNA, this 23S-enriched rRNA fraction was considered appropriate for the HPLC analysis.

For the HPLC analysis of nucleosides, 35 µg of bulk tRNA and 70 µg of 23S-enriched rRNA were hydrolyzed with nuclease P1 (Sigma) by overnight incubation in water with 1 mM ZnSO₄, followed by treatment with *E. coli* alkaline phosphatase (Sigma) at pH 8.3 for 2 h. The hydrolysate was analyzed by HPLC using a Develosil 5-µm C30 RP-Aqueous column (Phenomenex) with gradient elution to obtain optimal separations of nucleosides. Buffer A contained 2.5% methanol, 10 mM NH₄H₂PO₄, pH 5.1, while buffer B contained 25% methanol, 10 mM NH₄H₂PO₄, pH 5.3. The time for gradient elution was extended to 70 min. Nucleoside modifications were evaluated at a 260-nm wavelength using the EZchrom Elite software.

In vivo complementation

The *rlmN* open reading frame from *Escherichia coli* was amplified using the oligonucleotides: 5'-CGCCCATGGGTATCATCACCATCACCATTCTGAACAATTAGTCACACCTGAA and 3'-GCCG GATCCTTATCAGACCGCTTTAATGTCGATG encoding the NcoI and BamHI restriction sites (bold) and the N-terminal histidine tag (italics). The PCR product was digested and inserted into an NcoI/BamHI linearized pET15b plasmid by incubation with T4 ligase at 16°C overnight. The pET15b-*his-rlmN* construct was used to transform the BW25113 $\Delta rlmN$ strain; the empty pET15b plasmid was used to transform BW25113 wild-type and BW25113 $\Delta rlmN$ cells as controls. Bulk tRNA isolation from these plasmid-bearing strains and HPLC-nucleoside analysis were carried out as above.

Construction, purification, and in vivo analysis of tRNA^{Chimera}_{UUG} and its derivatives

Initially, the gene for tRNA^{Gln}_{cmnm5s2UUG} was inserted into the scaffold tRNA (at the EcoRV site) carried by pBSK_{RNA} (Ponchon et al. 2009). When we attempted to overexpress this construct in DH5α cells (as well as in MC4100, BW25113, or MG1655), following the recommended protocol (Ponchon et al. 2009), we were unable to observe accumulation of the construct from bulk

TABLE 3. List of strains and plasmids used in this study

Designation	Description	Construction	Origin
Strains			
BW25113	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3), λ ⁻ , <i>rph</i> -1, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514		(Datsenko and Wanner 2000)
DEV16	F ⁻ <i>thi-1 rel-1 spoT1 lacZ</i> 105 _{UAG} <i>val</i> ^R <i>mnmEQ</i> 192X		(Elseviers et al. 1984)
JC7623	<i>rpsL</i> 31 [<i>rpsL</i> K42T] ^a		(Bachmann 1996)
IC4639	DEV16 <i>mnmE</i> ⁺ <i>bgl</i>		(Yim et al. 2006)
IC5091	IC4639 <i>rpsL</i> K42T ^a	P1 (JC7623) × C4639	This study
IC5827	BW25113 <i>mnmE</i> :: <i>kan</i> ^b		(Baba et al. 2006)
IC5829	BW25113 <i>mnmA</i> :: <i>kan</i> ^b		(Baba et al. 2006)
IC5830	BW25113 <i>miaA</i> :: <i>kan</i> ^b		(Baba et al. 2006)
IC5937	IC4639 <i>mnmA</i> :: <i>kan</i> ^b	P1(IC5829) × IC4639	This study
IC5938	IC4639 <i>mnmE</i> :: <i>kan</i> ^b	P1(IC5827) × IC4639	This study
IC5940	IC4639 <i>miaA</i> :: <i>kan</i> ^b	P1(IC5830) × IC4639	This study
IC6464	BW25113 <i>rlmN</i> :: <i>kan</i> ^b		(Baba et al. 2006)
IC6625	MG1655 <i>acrF</i> ::Tn10 <i>rpsD</i> 12 ^c		(Ballesteros et al. 2001)
IC6626	IC4639 <i>rpsD</i> 12 ^c	P1 (IC6625) × IC4639	This study
IC6631	BW25113 <i>truA</i> :: <i>kan</i> ^b		(Baba et al. 2006)
IC6698	BW25113 <i>trmJ</i> :: <i>kan</i> ^b		(Baba et al. 2006)
IC6723	IC4639 <i>rlmN</i> :: <i>kan</i> ^b	P1(IC6464) × IC4639	This study
Plasmids			
pBSKra	Vector used for expression and purification of recombinant RNA		(Ponchon et al. 2009)
pET15b	Vector used for the production of C-terminally His-tagged proteins under T7 promoter control		Novagen
pIC1635	pET15b- <i>his-rlmN</i>		This study
pIC1666	tRNA ^{Chimera} _{UUG}		This study

^aThe *rpsL* mutation carried by JC7623 was identified by DNA sequencing in our laboratory. The mutation involves a change K42T in ribosomal protein S12.

^bAll the fusions to *kan* involve the deletion of the fused gene. Therefore, mutations *mnmE*::*kan*, *mnmA*::*kan*, *miaA*::*kan*, *rlmN*::*kan*, *truA*::*kan*, and *trmJ*::*kan* are named Δ*mnmE*, Δ*mnmA*, Δ*miaA*, Δ*rlmN*, Δ*truA*, and Δ*trmJ*, respectively, in the text.

^c*rpsD*12 is a deletion of nucleotides C528–A532 in ribosomal protein S4.

tRNA preparations analyzed by agarose gel electrophoresis (data not shown) or to recover any fraction by the Chaplet Column Chromatography method (Suzuki and Suzuki 2007). This also occurred with other RlmN tRNA substrates such as tRNA^{Arg}_{ICG}, tRNA^{Asp}_{QUC}, tRNA^{Glu}_{mnm5s2UUG}, and tRNA^{His}_{QUG}. Therefore, it seems that some chimera constructs are unstable and that they undergo degradation by native RNases. We finally constructed a tRNA^{Chimera}_{UUG} by replacing the ASL present in the tRNA scaffold with the ASL of tRNA^{Gln}_{cmnm5s2UUG} (see Fig. 4). This procedure was assisted by designing chimera constructs in the ssDNA version, followed by PCR amplification and cloning into an EcoRI/PstI linearized pBSKra plasmid. The resulting tRNA^{Chimera}_{UUG} was stably overproduced and could be purified by the standard method. Subsequently, several directed mutagenesis assays were done to introduce the following changes: A37C, U35A, U35G, G36U, and G36A. Using a similar procedure, we constructed truncated versions of tRNA^{Chimera}_{UUG} by deleting the D or the T stem-loops. However, these constructs were unstable since no accumulation was observed. All stable tRNA^{Chimera}_{UUG} versions were purified by the Chaplet Column Chromatography method (Suzuki and Suzuki 2007) with the DNA probe ^{biotin}5'-TGGCGCCCGAACAGGGACTTGAACCC, complementary to the scaffold human cytosolic tRNA^{Lys}₃ moiety of the tRNA^{Chimera}_{UUG}, and immobilized in a HiTrap Streptavidin HP column (GE Healthcare). Then, 25 μg of purified tRNA^{Chimera}_{UUG} (or its mutant

derivatives) was P1 hydrolyzed and analyzed by HPLC. Modifications s⁴U, D, m⁷G, T, and Ψ, reported to be present in the tRNA scaffold after in vivo overproduction in *E. coli* cells (Ponchon and Dardel 2007), were observed according to their UV spectra (spectra at 260 nm-wavelength range) and elution time (Gehrke and Kuo 1989). Nucleoside quantitation was obtained from the peak areas in the HPLC chromatograms with the EZchrom Elite software. Each nucleoside was normalized to the cytidine area, and the normalized data from mutants were referred to normalized data from the wild-type control.

Quantitation of tRNA^{Chimera}_{UUG} and derivatives

Quantitative Real-Time PCR (qRT-PCR) analysis was performed to measure the expression of tRNA^{Chimera}_{UUG} and derivatives in wild-type cells. Total RNA was isolated from cultures under identical growth conditions to those used for chimeric tRNA purification. Amplification of chimeric tRNAs and native tRNA^{Lys}_{mnm5s2UUU} was carried out using specific primers (Supplemental Table S1). qRT-PCR was performed in a one-step format using the SYBR Green PCR Master Mix (Applied Biosystems), 150 ng of total RNA, 5 units of MultiScribe Reverse Transcriptase (Applied Biosystems), and a StepOne Real Time PCR instrument (Applied Biosystems). Relative quantitation of chimeric tRNA expression was calculated with StepOne Software v2.0 (Applied Biosystems)

using the expression of native tRNA^{Lys}_{mnm5s2U} as the reference sample.

Determining RlmN in vitro activity on tRNA

Radical SAM proteins containing Fe-S clusters, pivotal for their activity, require further processing to allow a proper assembly of this chemical cluster in vitro (Pierrel et al. 2002; Yan et al. 2010). Recombinant RlmN enzyme has been previously overproduced to carry out in vitro methylations of 23S rRNA (Yan et al. 2010). To obtain an active RlmN protein, we overexpressed recombinant His-RlmN from pET15b-*his-rlmN* in ArcticExpress cells (Agilent Technologies) at 13°C in LB medium supplemented with 0.2 mM FeCl₃ and 0.5 mM IPTG for 24 h. His-RlmN was purified by affinity chromatography (TALON, Clontech) using 100 mM Tris-HCl pH 8.0, 250 mM NaCl buffer, which was previously degassed and extensively purged with N₂ to reduce O₂ levels (to 0.2–0.4 ppm) in the solution as described previously (Butler et al. 1994). The in vitro reconstitution of the His-RlmN Fe-S cluster was performed by also employing degassed and N₂ purged stock solutions. Purified His-RlmN was diluted in 1.5 mL of 100 mM Tris-HCl pH 7.5, 2 mM DTT, and incubated at 25°C with gentle shaking for 20 min. Then, 0.015 mL of FeCl₃ solution (2 mM DTT, 200 mM FeCl₃, and 10 mM cysteine) were added, and incubation with gentle shaking was allowed for a further 30-min period. Finally, the Fe-S cluster assembly was initiated by the addition of 0.2 mM Na₂S, and incubation was extended to 3–4 h with gentle shaking at 25°C. The protein solution was centrifuged for 5 min at 10,000g to remove precipitated Fe. Then, the supernatant was recovered, and His-RlmN was washed and concentrated in an Amicon device equipped with a 30-kDa MW cut off membrane (Millipore). Proper Fe-S assembly was noted by obtaining a brownish fraction of His-RlmN at the end of this process. To assay the RlmN methyltransferase activity in vitro, the reaction mix (degassed and N₂ purged) contained 100 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 2 mM sodium dithionite, 1 mM SAM, 15 µg of in vivo-transcribed tRNA^{Chimera}_{UUG} (obtained from *ΔrlmN* cells), and 40 µM His-RlmN. After 2 h at 37°C with gentle shaking, the methylation reaction was stopped by the addition of 1 vol of phenol pH 4.3, and tRNA contained in the aqueous phase was ethanol-precipitated, P1-digested, and HPLC-analyzed.

Readthrough assay

Strain IC4639 and derivatives contain a *lacZ* gene with an in-frame UAG stop codon. Misreading of this codon was quantitated by measuring β-galactosidase activity as previously described (Elseviers et al. 1984; Miller 1990) with minor modifications. Cultures were supplemented with 1.0 mM IPTG in order to enhance β-galactosidase activity. Samples for the assays were taken from exponentially growing cultures (OD₆₀₀ ~ 0.6) after at least 10 generations of steady-state growth. Data were compared for statistical significance using a *t*-test with Welch's correction.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank Dr. Luc Ponchon for the gift of the pBSK_{rna} plasmid. We also thank the National BioResource Project (NIG, Japan) and the *E. coli* genetic Stock Center (CGSC) for providing *E. coli* strains used in this study. This work has been supported by the Spanish Ministry of Science and Innovation (BFU2007-66509 and BFU2010-19737 grants) and the Generalitat Valenciana (APE-010/11 and ACOMP/2012/065) to M.E.-A., and a PhD fellowship from the Prince Felipe Research Centre to A.B.-P.

Received March 15, 2012; accepted June 26, 2012.

REFERENCES

- Agris PF. 1996. The importance of being modified: Roles of modified nucleosides and Mg²⁺ in RNA structure and function. *Prog Nucleic Acid Res Mol Biol* **53**: 79–129.
- Agris PF. 2004. Decoding the genome: A modified view. *Nucleic Acids Res* **32**: 223–238.
- Agris PF. 2008. Bringing order to translation: The contributions of transfer RNA anticodon-domain modifications. *EMBO Rep* **9**: 629–635.
- Agris PF, Vendex FA, Graham WD. 2007. tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol* **366**: 1–13.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.
- Andersen TE, Porse BT, Kirpekar F. 2004. A novel partial modification at C2501 in *Escherichia coli* 23S ribosomal RNA. *RNA* **10**: 907–913.
- Atta M, Mulliez E, Arragain S, Forouhar F, Hunt JF, Fontecave M. 2010. S-adenosylmethionine-dependent radical-based modification of biological macromolecules. *Curr Opin Struct Biol* **20**: 684–692.
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* **2**: 2006.0008. doi: 10.1038/msb4100050.
- Bachmann D. 1996. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In *Escherichia coli and Salmonella: Cellular and molecular biology* (ed. F Neidhardt, R Curtis), pp. 2460–2488. American Society for Microbiology, Washington, DC.
- Ballesteros M, Fredriksson A, Henriksson J, Nystrom T. 2001. Bacterial senescence: Protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. *EMBO J* **20**: 5280–5289.
- Benítez-Páez A, Villarroja M, Douthwaite S, Gabaldon T, Armengod ME. 2010. YibK is the 2'-O-methyltransferase TrmL that modifies the wobble nucleotide in *Escherichia coli* tRNA^{Leu} isoacceptors. *RNA* **16**: 2131–2143.
- Björk GR, Hagervall TG. 2005. Transfer RNA modification. In *EcoSal—Escherichia coli and Salmonella: Cellular and molecular biology* (ed. RCI Böck et al.). ASM Press, Washington, DC.
- Björk GR, Jacobsson K, Nilsson K, Johansson MJ, Bystrom AS, Persson OP. 2001. A primordial tRNA modification required for the evolution of life? *EMBO J* **20**: 231–239.
- Butler IB, Schoonen MA, Rickard DT. 1994. Removal of dissolved oxygen from water: A comparison of four common techniques. *Talanta* **41**: 211–215.
- Chimnarong S, Forouhar F, Sakai J, Yao M, Tron CM, Atta M, Fontecave M, Hunt JF, Tanaka I. 2009. Snapshots of dynamics in synthesizing N⁶-isopentenyladenosine at tRNA anticodon. *Biochemistry* **48**: 5057–5065.

- Cho KH, Caparon MG. 2008. tRNA modification by GidA/MnmE is necessary for *Streptococcus pyogenes* virulence: A new strategy to make live attenuated strains. *Infect Immun* **76**: 3176–3186.
- Chow CS, Lamichhane TN, Mahto SK. 2007. Expanding the nucleotide repertoire of the ribosome with post-transcriptional modifications. *ACS Chem Biol* **2**: 610–619.
- Czerwoniec A, Dunin-Horkawicz S, Purta E, Kaminska KH, Kasprzak JM, Bujnicki JM, Grosjean H, Rother K. 2009. MODOMICS: A database of RNA modification pathways. 2008 update. *Nucleic Acids Res* **37**: D118–D121.
- Dao V, Guenther R, Malkiewicz A, Nawrot B, Sochacka E, Kraszewski A, Jankowska J, Everett K, Agris PF. 1994. Ribosome binding of DNA analogs of tRNA requires base modifications and supports the “extended anticodon”. *Proc Natl Acad Sci* **91**: 2125–2129.
- Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci* **97**: 6640–6645.
- Decatur WA, Fournier MJ. 2002. rRNA modifications and ribosome function. *Trends Biochem Sci* **27**: 344–351.
- Do CB, Mahabhashyam MS, Brudno M, Batzoglou S. 2005. ProbCons: Probabilistic consistency-based multiple sequence alignment. *Genome Res* **15**: 330–340.
- Eddy SR. 1998. Profile hidden Markov models. *Bioinformatics* **14**: 755–763.
- El Yacoubi B, Lyons B, Cruz Y, Reddy R, Nordin B, Agnelli F, Williamson JR, Schimmel P, Swairjo MA, de Crecy-Lagard V. 2009. The universal YrdC/Sua5 family is required for the formation of threonylcarbamoyladenosine in tRNA. *Nucleic Acids Res* **37**: 2894–2909.
- Elseviers D, Petruccio LA, Gallagher PJ. 1984. Novel *E. coli* mutants deficient in biosynthesis of 5-methylaminomethyl-2-thiouridine. *Nucleic Acids Res* **12**: 3521–3534.
- Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE, Rhee HW, Hong JJ, Hartland EL, et al. 2010. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. *PLoS Pathog* **6**: e1000944. doi:10.1371/journal.ppat.1000944.
- Gehrke CW, Kuo KC. 1989. Ribonucleoside analysis by reversed-phase high-performance liquid chromatography. *J Chromatogr* **471**: 3–36.
- Giessing AM, Jensen SS, Rasmussen A, Hansen LH, Gondela A, Long K, Vester B, Kirpekar F. 2009. Identification of 8-methyladenosine as the modification catalyzed by the radical SAM methyltransferase Cfr that confers antibiotic resistance in bacteria. *RNA* **15**: 327–336.
- Golovina AY, Sergiev PV, Golovin AV, Serebryakova MV, Demina I, Govorun VM, Dontsova OA. 2009. The *yfiC* gene of *E. coli* encodes an adenine-N6 methyltransferase that specifically modifies A37 of tRNA^{Val}(cmo⁵UAC). *RNA* **15**: 1134–1141.
- Gong S, Ma Z, Foster JW. 2004. The Era-like GTPase TrmE conditionally activates *gadE* and glutamate-dependent acid resistance in *Escherichia coli*. *Mol Microbiol* **54**: 948–961.
- Gray J, Wang J, Gelvin SB. 1992. Mutation of the *miaA* gene of *Agrobacterium tumefaciens* results in reduced *vir* gene expression. *J Bacteriol* **174**: 1086–1098.
- Grosjean H. 2005. Fine tuning of RNA functions by modification and editing. In *Topics in current genetics* (ed. S Hohmann). Springer Verlag, New York, NY.
- Grove TL, Benner JS, Radle MI, Ahlum JH, Landgraf BJ, Krebs C, Booker SJ. 2011. A radically different mechanism for S-adenosyl-methionine-dependent methyltransferases. *Science* **332**: 604–607.
- Havelund JF, Giessing AMB, Hansen T, Rasmussen A, Scott LG, Kirpekar F. 2011. Identification of 5-hydroxycytidine at position 2501 concludes characterization of modified nucleosides in *E. coli* 23S rRNA. *J Mol Biol* **411**: 529–536.
- Jenner L, Demeshkina N, Yusupova G, Yusupov M. 2010. Structural rearrangements of the ribosome at the tRNA proofreading step. *Nat Struct Mol Biol* **17**: 1072–1078.
- Kaminska KH, Purta E, Hansen LH, Bujnicki JM, Vester B, Long KS. 2010. Insights into the structure, function, and evolution of the radical-SAM 23S rRNA methyltransferase Cfr that confers antibiotic resistance in bacteria. *Nucleic Acids Res* **38**: 1652–1663.
- Kimura S, Suzuki T. 2010. Fine-tuning of the ribosomal decoding center by conserved methyl-modifications in the *Escherichia coli* 16S rRNA. *Nucleic Acids Res* **38**: 1341–1352.
- Kurland C, Hughes D, Ehrenberg M. 1996. Limitations of translational accuracy. In *Escherichia coli and Salmonella: Cellular and molecular biology* (ed. F Neidhardt et al.), pp. 979–1004. ASM Press, Washington, DC.
- Lamarre JM, Howden BP, Mankin AS. 2011. Inactivation of the indigenous methyltransferase RlmN in *Staphylococcus aureus* increases linezolid resistance. *Antimicrob Agents Chemother* **55**: 2989–2991.
- Li JN, Bjork GR. 1995. 1-Methylguanosine deficiency of tRNA influences cognate codon interaction and metabolism in *Salmonella typhimurium*. *J Bacteriol* **177**: 6593–6600.
- Miller JH. 1990. *A short course in bacterial genetics: A laboratory manual and handbook for Escherichia coli and related bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Nasvall SJ, Chen P, Bjork GR. 2004. The modified wobble nucleoside uridine-5-oxyacetic acid in tRNA^{Pro}(cmo⁵UGG) promotes reading of all four proline codons in vivo. *RNA* **10**: 1662–1673.
- Nasvall SJ, Chen P, Bjork GR. 2007. The wobble hypothesis revisited: Uridine-5-oxyacetic acid is critical for reading of G-ending codons. *RNA* **13**: 2151–2164.
- Numata T, Ikeuchi Y, Fukai S, Suzuki T, Nureki O. 2006. Snapshots of tRNA sulphuration via an adenylated intermediate. *Nature* **442**: 419–424.
- Petruccio LA, Gallagher PJ, Elseviers D. 1983. The role of 2-methylthio-N6-isopentenyladenosine in readthrough and suppression of nonsense codons in *Escherichia coli*. *Mol Gen Genet* **190**: 289–294.
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* **25**: 1605–1612.
- Pierrel F, Bjork GR, Fontecave M, Atta M. 2002. Enzymatic modification of tRNAs: MiaB is an iron-sulfur protein. *J Biol Chem* **277**: 13367–13370.
- Ponchon L, Dardel F. 2007. Recombinant RNA technology: The tRNA scaffold. *Nat Methods* **4**: 571–576.
- Ponchon L, Beauvais G, Nonin-Lecomte S, Dardel F. 2009. A generic protocol for the expression and purification of recombinant RNA in *Escherichia coli* using a tRNA scaffold. *Nat Protoc* **4**: 947–959.
- Pruitt KD, Tatusova T, Brown GR, Maglott DR. 2012. NCBI Reference Sequences (RefSeq): Current status, new features and genome annotation policy. *Nucleic Acids Res* **40**: D130–D135.
- Rafferty LA, Yarus M. 1987. Systematic alterations in the anticodon arm make tRNA^{Glu}-Su_{oc} a more efficient suppressor. *EMBO J* **6**: 1499–1506.
- Ramu H, Vazquez-Laslop N, Klepacki D, Dai Q, Piccirilli J, Micura R, Mankin AS. 2011. Nascent peptide in the ribosome exit tunnel affects functional properties of the A-site of the peptidyl transferase center. *Mol Cell* **41**: 321–330.
- Sha J, Kozlova EV, Fadl AA, Olano JP, Houston CW, Peterson JW, Chopra AK. 2004. Molecular characterization of a glucose-inhibited division gene, *gida*, that regulates cytotoxic enterotoxin of *Aeromonas hydrophila*. *Infect Immun* **72**: 1084–1095.
- Suzuki T, Suzuki T. 2007. Chaplet column chromatography: Isolation of a large set of individual RNAs in a single step. *Methods Enzymol* **425**: 231–239.
- Toh SM, Xiong L, Bae T, Mankin AS. 2008. The methyltransferase YfgB/RlmN is responsible for modification of adenosine 2503 in 23S rRNA. *RNA* **14**: 98–106.

- Urbonavicius J, Qian Q, Durand JM, Hagervall TG, Bjork GR. 2001. Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J* **20**: 4863–4873.
- Vallabhaneni H, Farabaugh PJ. 2009. Accuracy modulating mutations of the ribosomal protein S4-S5 interface do not necessarily destabilize the rps4-rps5 protein–protein interaction. *RNA* **15**: 1100–1109.
- van Buul CP, Visser W, van Knippenberg PH. 1984. Increased translational fidelity caused by the antibiotic kasugamycin and ribosomal ambiguity in mutants harbouring the *ksgA* gene. *FEBS Lett* **177**: 119–124.
- Vazquez-Laslop N, Ramu H, Klepacki D, Kannan K, Mankin AS. 2010. The key function of a conserved and modified rRNA residue in the ribosomal response to the nascent peptide. *EMBO J* **29**: 3108–3117.
- Yan F, Fujimori DG. 2011. RNA methylation by radical SAM enzymes RlmN and Cfr proceeds via methylene transfer and hydride shift. *Proc Natl Acad Sci* **108**: 3930–3934.
- Yan F, LaMarre JM, Rohrich R, Wiesner J, Jomaa H, Mankin AS, Fujimori DG. 2010. RlmN and Cfr are radical SAM enzymes involved in methylation of ribosomal RNA. *J Am Chem Soc* **132**: 3953–3964.
- Yarus M. 1982. Translational efficiency of transfer RNA's: Uses of an extended anticodon. *Science* **218**: 646–652.
- Yarus M, Cline S, Raftery L, Wier P, Bradley D. 1986. The translational efficiency of tRNA is a property of the anticodon arm. *J Biol Chem* **261**: 10496–10505.
- Yim L, Martínez-Vicente M, Villarroja M, Aguado C, Knecht E, Armengod ME. 2003. The GTPase activity and C-terminal cysteine of the *Escherichia coli* MnmE protein are essential for its tRNA modifying function. *J Biol Chem* **278**: 28378–28387.
- Yim L, Moukadiri I, Bjork GR, Armengod ME. 2006. Further insights into the tRNA modification process controlled by proteins MnmE and GidA of *Escherichia coli*. *Nucleic Acids Res* **34**: 5892–5905.

RNA

A PUBLICATION OF THE RNA SOCIETY

The *Escherichia coli* RlmN methyltransferase is a dual-specificity enzyme that modifies both rRNA and tRNA and controls translational accuracy

Alfonso Benítez-Páez, Magda Villarroya and M.-Eugenia Armengod

RNA 2012 18: 1783-1795 originally published online August 13, 2012
Access the most recent version at doi:[10.1261/rna.033266.112](https://doi.org/10.1261/rna.033266.112)

Supplemental Material <http://rnajournal.cshlp.org/content/suppl/2012/07/25/rna.033266.112.DC1>

References This article cites 60 articles, 26 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/18/10/1783.full.html#ref-list-1>

License

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
<http://rnajournal.cshlp.org/subscriptions>