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Radical SAM-Mediated Methylation of Ribosomal RNA

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Abstract

Post-transcriptional modifications of RNA play an important role in a wide range of biological processes. In ribosomal RNA (rRNA), methylation of nucleotide bases is the predominant modification. In recent years, methylation of adenosine 2503 (A2503) in bacterial 23S rRNA has attracted significant attention due to both the unusual regioselectivity of the methyl group incorporation, as well as the pathophysiological roles of the resultant methylations. Specifically, A2503 is methylated at the C2 and C8 positions of the adenine ring, and the introduced modifications have a profound impact on translational fidelity and antibiotic resistance, respectively. These modifications are performed by RlmN and Cfr, two members, of the recently discovered class of radical S-adenosylmethionine (radical SAM) methylsynthases. Here, we present several methods that can be used to evaluate the activity of these enzymes, under both *in vivo* and *in vitro* conditions.

Keywords

Radical SAM methylation; RlmN; Cfr; Ribosomal RNA; 2-Methyladenosine; 8-Methyladenosine

1 Introduction

Post-transcriptional modifications of nucleotides play an important and ubiquitous, yet incompletely understood role in proper RNA function. To date, more than 100 different types of modifications have been reported for various RNA molecules, including ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), small nuclear (snRNA), and microRNA (miRNA) (Machnicka et al., 2013). These modifications are chemically diverse and are comprised of methylation, hydroxylation, acetylation, deamination, isomerization, selenylation, reduction, and cyclization, to name a few (Agris, 1996). From this wide variety of modifications, methylation of nucleotides, both at nucleobases and at the ribose moiety, are one of the most common and simplest modifications, and the most prevalent modification in rRNA (Sergeeva, Bogdanov, & Sergiev, 2014). Most of the methylation sites are located in the functional centers of the ribosome: in the decoding and peptidyl transferase centers, as well as on the interface of ribosomal subunits. The roles of

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such modifications are not completely understood; however, their conservation across bacterial species implies their functional importance. It is believed that their roles span from structural stabilization of substrate RNA to modulation of the function of the ribosome (e.g., maintenance of translational fidelity) (Li & Mason, 2014; Motorin & Helm, 2011; Sergeeva et al., 2014; Sergiev et al., 2011). Additionally, post-transcriptional modification of rRNA can also confer antibiotic resistance toward ribosome-targeting antibiotics (Leclercq & Courvalin, 1991; Long, Poehlsgaard, Kehrenberg, Schwarz, & Vester, 2006; McCusker & Fujimori, 2012; Poehlsgaard & Douthwaite, 2005; Vester & Long, 2009; Wilson, 2014).

Several classes of RNA methylating enzymes perform methylation of rRNA. These enzymes are mechanistically diverse and their mode of methylation depends on the electronic demands of the substrate (Motorin & Helm, 2011). Nucleophilic sites in nucleotides are modified by methyltransferases that transfer methyl group from *S*-adenosylmethionine (SAM) to nucleophilic atoms (e.g., heteroatoms of nucleobases and hydroxyl groups in the ribose) via an S_N2 displacement mechanism (e.g., Dunkle et al., 2014; Hansen, Kirpekar, & Douthwaite, 2001; Lövgren & Wikström, 2001; Tscherne, Nurse, Popienick, & Ofengand, 1999). Another mechanistically distinct type of RNA methyltransferases is enzymes that perform methylation of cytosine and uridine at the C5 carbon. These enzymes activate their substrates via Michael addition of the catalytic Cys to C6 carbon on the pyrimidine ring. The formed adduct activates the C5 position for a nucleophilic attack on the methyl group of SAM. Subsequent elimination of the Cys moiety leads to the formation of C5-methylated cytosines and uridines (e.g., Agarwalla, Stroud, & Gaffney, 2004; King & Redman, 2002). Unique among rRNA methylating enzymes are those that methylate the C2 and C8 amidine carbons of adenosine 2503 (A2503). Recent studies by our lab and by others (Boal et al., 2011; Grove et al., 2011, 2013; McCusker et al., 2012; Silakov et al., 2014; Yan & Fujimori, 2011; Yan et al., 2010) have unraveled a mechanistically novel type of RNA methylation, catalyzed by radical SAM methylsynthases RlmN and Cfr. Unlike methyltransferases, these enzymes incorporate only a methylene fragment, ultimately derived from the methyl group of SAM, into the methyladenosine product. In this chapter, we describe in detail several procedures used in our lab to detect the activity of RlmN and Cfr both *in vivo* and *in vitro* (Fig. 1). Although we will use RlmN/Cfr as our model systems, the methodology presented herein can be applied to study various types of RNA methylations. As 23S rRNA is a shared substrate for both RlmN and Cfr, this chapter will focus on methods implemented in our lab to examine the activity of these enzymes toward 23S rRNA.

2 Mechanism of RNA Methylation by RlmN and Cfr

RlmN and Cfr belong to the radical SAM superfamily. A hallmark of this family is characteristic and highly conserved CX_3CX_2C motif that ligates the [4Fe-4S] iron-sulfur cluster (Sofia, Chen, Hetzler, Reyes-Spindola, & Miller, 2001). This iron-sulfur cluster serves as a cofactor and its reduction is required for initiation of the reaction. Both RlmN and Cfr assemble a methyl group on their products from a hydrogen atom initially present on the substrate and a methylene fragment ultimately derived from SAM. Experimental evidence suggests a shared mechanism among these enzymes (Fig. 2) (Boal et al., 2011; Grove et al., 2011, 2013; McCusker et al., 2012; Silakov et al., 2014; Yan & Fujimori, 2011). Their mutual substrate is a single adenosine nucleotide—A2503 of 23S rRNA

(*Escherichia coli* numbering). RlmN from *E. coli* is known to also methylate a subset of tRNAs *in vivo* (Benitez-Paez, Villarroya, & Armengod, 2012). Several lines of evidence indicate that their reaction requires two SAM molecules. In the first-step of the reaction, one of the conserved Cys residues (C355, *E. coli* RlmN numbering) is methylated by SAM in an S_N2 displacement reaction. Following methylation of C355, the reduced [4Fe–4S] cluster catalyzes the reductive cleavage of the second SAM molecule producing a 5'-deoxyadenosyl (5'-dA•) radical. The 5'-dA• abstracts a hydrogen from a methyl group on C355 producing a cysteine-bound methylene radical. In the subsequent step, this methylene radical adds to the amidine carbon on the adenine ring (C2 position for RlmN; C8 for Cfr), resulting in an enzyme–RNA adduct. This adduct has both been isolated by mutagenesis (McCusker et al., 2012) and characterized spectroscopically (Silakov et al., 2014). The adduct is resolved by a second conserved cysteine, C118, which initiates the fragmentation of the enzyme–RNA crosslink to release the enzyme and to form the methylated RNA product (Boal et al., 2011; Grove et al., 2011; McCusker et al., 2012; Silakov et al., 2014).

3 Expression of RlmN and Cfr

3.1 Protocol for Expression of His₆-Tagged RlmN and Cfr Proteins in LB Medium

The *E. coli* strain BL21(DE3) is cotransformed with pET21a-RlmN (or pET15b-Cfr) with pDB1282, a plasmid that encodes proteins required for the biogenesis of the iron–sulfur clusters and their incorporation into target enzymes. This bacterial transformation is plated on LB agar containing 100 µg/ml ampicillin and 50 µg/ml kanamycin, and incubated at 37 °C. A single colony is used to inoculate 50 ml of LB medium containing antibiotics, which is then incubated overnight at 37 °C, 180 rpm. Four flasks with 1 l of LB medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin are inoculated with 10 ml of the overnight culture and cultivated at 37 °C until they reach OD₆₀₀ of 0.3–0.4. At this point, expression of the *isc* operon from pDB1282 is initiated via addition of 20% (w/v) arabinose solution to a final concentration of 0.2%. Next, FeCl₃ (200 µM) and l-cysteine (200 mM) are added drop-wise to a final concentration of 200 µM of each. Flasks are cooled on ice while the temperature of the incubator is reduced to 18 °C. When the content is cooled to ~ 20 °C, cultures are returned to an incubator and left to shake until OD₆₀₀ reaches 0.6–0.8. At that point, the production of RlmN is induced by the addition of isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 200 µM, and the bacterial culture is incubated at 18 °C for an additional 24 h. Cells are harvested by centrifugation at 4000 rpm for 20 min at 4 °C. Due to the presence of Fe³⁺ and S²⁻ in the LB medium during the expression, as well as enzymes responsible for the biogenesis of [4Fe–4S] cluster, RlmN overexpressed by this protocol is methylated at C355 (C338 in *Staphylococcus aureus* Cfr) (Boal et al., 2011; Grove et al., 2011, 2013; McCusker et al., 2012).

3.2 Protocol for the Expression of apo His₆-Tagged RlmN and Cfr Proteins in Minimal Medium

A single colony of the BL21(DE3) *E. coli* cells containing pET21a-RlmN or pET15b-Cfr is used to inoculate 50 ml of LB medium containing 100 µg/ml ampicillin, which is left to incubate overnight at 37 °C with shaking at 180 rpm. The following day, each of four 1.35 l of M9 minimal medium (Sigma–Aldrich) containing 150 µl of 1 M CaCl₂, 3 ml of 1 M

MgSO₄, 30 ml of 1 M glucose, and 1.5 ml of 100 mg/ml Amp is inoculated with 15 ml of the overnight culture. Flasks are incubated at 37 °C, 180 rpm until OD₆₀₀ reaches 0.3, at which point 150 ml of 5×EZ mix amino acids (Teknova) is added. At an OD₆₀₀ of ~ 0.6, cultures are cooled on ice to ~ 20 °C, while the temperature of the incubator is reduced to 18 °C. Fifteen milliliters of 7.5 mM 1,10-phenanthroline (Sigma–Aldrich) and 1.5 ml of 200 mM IPTG are added into each flask, followed by the incubation at 18 °C for 24 h. Cells are harvested by centrifugation at 4000 rpm for 20 min at 4 °C. Then cells are washed and repelleted twice with 1 × PBS (pH 7.2) cold solution to remove any residual phenanthroline. This protocol allows for the production of apo RlmN and Cfr. As Fe³⁺ and S²⁻ are absent under these conditions, the [4Fe–4S] cluster is not assembled. As a result C355 (*E. coli* RlmN) and corresponding C338 (*S. aureus* Cfr) are unmodified.

4 Purification of RlmN and Cfr

Due to the high sensitivity of the [4Fe–4S] cluster to oxygen, purification of these proteins is carried out under strict anaerobic conditions. Anaerobic conditions are maintained during purification of both iron–sulfur containing and apo-RlmN and Cfr in order to prevent any oxygen contamination in downstream applications. The temperature of the anaerobic chamber (MBraun) is kept at ~ 10 °C, and oxygen levels are maintained below 1.8 ppm.

Lysis buffer (50 mM HEPES pH 7.5, 300 mM KCl, 2 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol), wash buffer (50 mM HEPES pH 7.5, 300 mM KCl, 10 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol), elution buffer (50 mM HEPES pH 7.5, 300 mM KCl, 250 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol), 2 × reconstitution buffer (200 mM HEPES, pH 7.5, 1 M KCl, 20% glycerol), and gel-filtration buffer (10 mM HEPES, pH 7.5, 500 mM KCl, 10% glycerol, 5 mM DTT) are all prepared in advance and deoxygenated by bubbling under argon for at least 1 h prior to being introduced into the anaerobic chamber, where they are left to equilibrate overnight.

For purification, 10–15 g of cell pellets are brought into the anaerobic chamber and resuspended in 35–40 ml of the lysis buffer. Cells are then transferred into an air-tight sonicator cell and lysed outside of the anaerobic chamber. Sonication is performed on ice at 50% duty cycle using a Branson 450 Sonifier sonic dismembrator (Fisher). Power output is set to 5–7, with 1 min duration of each pulse followed by sufficient cooling of the sonicator cell between each sonic disruption. The sonicator cell is then returned to the glove box, where the lysate is poured into centrifuge tubes. The cells are centrifuged at 35,000 rpm, 4 °C, for 45 min. Meanwhile, ~ 7–8 ml of 50% slurry of Talon cobalt resin (Clontech) is prepared by washing the resin with distilled deionized water (ddI H₂O) 3 times, resuspension in ~ 10 ml of ddI H₂O and deoxygenation under argon for ~ 45 min. After centrifugation, the centrifuge tubes together with the Talon resin and 5 g of desalting P6 resin (BioRad—BioGel-P6DG Gel) are reintroduced into the glove box where the supernatant is carefully decanted into a clean Erlenmeyer flask. Talon resin is added to the supernatant and this suspension is left to equilibrate for 1 h with gentle stirring. After 1 h, the supernatant-resin suspension is applied to a column, after which the column content is washed with 50 ml of the lysis buffer followed by 25 ml of the wash buffer. The enzyme is eluted with 10 ml of elution buffer and collected in 1 ml fractions. Typically, coexpression

of the enzymes with pDB1282 results in fractions that contain significant brown color, while the enzymes expressed in the minimal medium are colorless. Protein-containing fractions, as identified by the Bradford method, are pooled and desalted on manually prepared P6 desalting column, and protein is eluted with 1 × reconstitution buffer. After desalting, protein concentration is adjusted to ~ 50–60 μM , 1 M DTT is added to a final concentration of 200 mM , and enzyme is left to equilibrate for 1 h. The reconstitution of the [4Fe–4S] cluster is then carried out by addition of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (both at 200 mM stock concentration). Iron solution is added drop-wise to a slowly stirred enzyme solution over a 30 min period, to a final concentration of 500 μM . The resulting solution is stirred for an additional 10 min prior to the drop-wise addition of the sulfide solution over 90 min, to a final concentration of 600 μM . The resulting dark brown solution is gently stirred for an additional 15 h. Following incubation, the reconstitution mixture is transferred to an ultracentrifuge tube, sealed inside of a glove box, and centrifuged at 30,000 rpm, 4 °C for 60 min. The tube is then brought into the glove box, and supernatant is desalted on the P6 resin (manually poured) in the gel filtration buffer. After desalting, the reconstituted enzyme is concentrated and flash frozen in liquid N_2 , before being stored at – 80 °C. To remove adventitiously bound iron and sulfide as well as aggregated protein from chemically reconstituted RlmN and Cfr, enzymes are further purified by gel filtration on S200 column (GE Healthcare) in gel filtration buffer. If needed, the purity of the enzyme can be further increased by anion exchange chromatography using Hi-TrapQ column (GE Healthcare) (Yan et al., 2010). It is important to note that the iron–sulfur cluster reconstitution step is performed not only for the apo enzymes, but also for enzymes coexpressed with iron–sulfur cluster assembly machinery in the LB medium—conditions that typically result in only partial reconstitution of the cluster.

5 *In Vitro* Methylation Assays for RlmN and Cfr

5.1 General Overview of *In Vitro* Methylation Assays

The standard *in vitro* methylation assay for RNA methylating enzymes measures the incorporation of radioactively labeled methyl group (e.g., ^{14}C or ^3H) from SAM into the RNA product. The resulting RNA product is isolated by phenol/chloroform extractions or by using a commercial kit, and radioactivity incorporation is measured by liquid scintillation counter (LSC). Alternatively, upon quenching of the reaction, reaction mixture is spotted onto a filter paper, unreacted radiolabeled SAM removed by washing the paper with trichloroacetic acid (TCA, 5%), and the filter paper subsequently transferred into vials containing scintillation liquid. The amount of transferred methyl group is quantified with LSC. Evaluation of the radioactivity allows for determination of molar amount of modified nucleotide within the RNA molecule. Additionally, a time course analysis allows for determination of the rate of a reaction (Chervin, Kittendorf, & Garcia, 2007).

A choice between ^{14}C and ^3H is solely on the researcher. In comparison to ^{14}C , tritium offers ~ 1000-fold higher specific radioactivity allowing for higher sensitivity of detection in LSC. In addition, due to a higher specific radioactivity, a broader range of final SAM concentrations can be used in the experiments. In contrast, ^{14}C has higher counting efficiency than tritium, and events such as chemiluminescence do not significantly interfere

with counting. Both compounds, [^{14}C -methyl]-SAM and [^3H -methyl]-SAM, are readily commercially available and can be purchased with a range of specific radioactivities (Perkin-Elmer).

In an alternative approach, activity of an RNA methylating enzyme can be assessed by the use of nonradioactive SAM, and subsequent detection of the product by liquid chromatography/mass spectrometry (LC/MS). In short, following the incubation of RNA with an enzyme and SAM, methylated RNA is isolated from the reaction mixture and digested to single nucleosides. The resulting nucleosides are then separated on HPLC and analyzed by LC/MS. While this approach allows for isolation of the product and analysis of its molecular weight, a disadvantage of this method is its lower sensitivity as compared to the radioactivity-incorporation-based method.

Regardless of method used for product analysis, it is important to indicate that one of the byproducts of the reaction, *S*-adenosylhomocysteine (SAH) is known to act as a competitive inhibitor of many methyltransferases (Clarke & Banfield, 2001). However, in most assays, including those utilized in our lab, SAM is present in a large excess over the substrate and therefore the amount of SAH produced during the course of the reaction should not cause significant inhibition. Nonetheless, as with any enzymatic assay, once the activity of RNA methylating enzyme is determined *in vitro*, it is important to optimize the experimental conditions prior to more systematic study of the mechanism and specificity of the enzyme. These include optimization of the reductant, type, and concentration of specific metal ions (e.g., Mg^{2+} for RNA substrates), the pH and the composition of the reaction buffer, etc. Moreover, strict nuclease-free conditions must be maintained in the experiment, and in some instances addition of RNase inhibitors is required. In our group, both RNase inhibitor from human placenta (New England BioLabs) and RNasin (Promega) have been used with equal success. Lastly, the stability of SAM needs to be taken into account, since the rate of SAM hydrolysis increases with an increase in pH and temperature.

5.2 Protocol for Substrate Preparation

When isolated from wild-type *E. coli* cells, naturally occurring RNAs are fully modified and as such are not suitable for testing *in vitro* activities of RNA methylating enzymes. While this challenge can be overcome by using knockout strains (e.g., BW2113/ *rlmV* strain, see below; Yan et al., 2010), an alternative approach relies on *in vitro* transcription of RNA fragments. When working with smaller RNAs, such as tRNAs that are ~ 75 -nt long, the whole biomolecule is usually transcribed. However, when working with longer substrates such as 23S rRNA, transcription of smaller fragments is preferred, as they are easier to prepare and handle than the full 23S rRNA. As the common substrate for RlmN and Cfr is 23S rRNA, here we describe *in vitro* transcription of 23S rRNA fragments that encompass the substrate nucleotide, A2503. In our earlier investigations, we have determined substrate requirements (i.e., length of the transcribed rRNA fragment) for the methylation of *in vitro* transcribed rRNA by RlmN and Cfr (Yan et al., 2010).

The transcription reactions for any fragment of rRNA can be carried out by following this protocol where the only difference is changing the primers used in the PCR amplification step. *In vitro* transcription reactions of 23S rRNA fragments are carried out in the following

manner: in the first step, a desired fragment of the DNA template is amplified using a plasmid pKK3535 that encodes a 23S rRNA gene (Yan et al., 2010). The polymerase that we utilize in this PCR step is Phusion High-Fidelity DNA polymerase (New England BioLabs); however, other polymerases can be used as well. The resultant PCR products are purified using the Qiagen PCR purification kit following the manufacturer's recommendations.

In a typical *in vitro* transcription reaction, 20 µg of DNA template is dissolved in 500 µl solution containing T7 buffer (40 mM Tris-HCl, pH 7.9, 16 mM MgCl₂, 2 mM spermidine), 20 mM DTT, 3.2 mM of each of rNTP, 0.2 U/µl RNase inhibitor from human placenta, and 3 U/µl T7 RNA polymerase (New England BioLabs). The reaction mixture is incubated at 37 °C for 3 h, followed by the addition of 40 U of RQ1 RNase Free DNase (Promega) and further incubation at 37 °C for 30 min. The transcribed rRNA is purified by RNeasy midi kit (Qiagen) following the manufacturer's recommendation. RNA is then precipitated overnight at – 20 °C by addition of 1/10th volume of 3 M NaOAc, pH 5 and 3 volumes of ethanol. The sample is subsequently pelleted, washed with 70% aqueous ethanol, air-dried, resuspended in RNase-free water, and stored at – 20 °C. The size and the purity of the resulting rRNA fragments are verified by gel electrophoresis on denaturing 4.5% TBE, 7 M urea-PAGE gel.

5.3 Protocol for Testing the Activities of RlmN and Cfr Using Total Radioactivity Incorporation

A typical activity assay for RlmN and Cfr is performed in 100 µl of HEPES buffer (100 mM, pH 8.0) containing 100 mM KCl, 10 mM MgCl₂, and 2 mM DTT to which 20 µM Flavodoxin (Flv), 2 µM Flavodoxin reductase (Flx), 0.14 µCi [¹⁴C-methyl]-SAM (58 mCi/mmol), 1 mM NADPH, 1 µM RlmN (or 2 µM Cfr), and 2 µM rRNA fragment are added. Alternatively, [³H-methyl]-SAM can be used as a source of radiolabeled SAM. All of the components except the radiolabeled SAM are made anaerobic by purging with argon prior to mixing in an MBraun glove box. The reaction mixture containing rRNA, enzyme, Flv, Flx, and the buffer is prepared, and subsequently removed from the anaerobic chamber in a gas-tight glass vials (Supelco). The content of the vial is kept under a positive pressure of argon by attachment of an argon balloon through a septum. The vials are kept on ice until the commencement of the reaction. [¹⁴C-methyl]-SAM (or [³H-methyl]-SAM) is supplied in a 9:1 mixture of 10 mM H₂SO₄:EtOH, which needs to be removed prior to reaction. The radiolabeled SAM is aliquoted into an empty glass vial (Supelco) and dried by an argon stream. The vial is then capped with a septum and maintained under a positive pressure of argon. The dried SAM is dissolved in 4 × buffer (400 mM HEPES pH 8, 400 mM KCl) added by a gas-tight syringe. To the content of this vial, the reaction mixture lacking NADPH is added by a gas-tight syringe, and the resulting mixture is left to equilibrate at 37 °C for 10 min under a positive pressure of argon. The reaction is then initiated by the addition of an anaerobic solution of NADPH (5 µl of 20 mM stock solution, final concentration of 1 mM). An initial sample (5 µl, *t* = 0 min) is immediately removed. This sample is used to determine the total amount of the radioactivity in the vial to account/correct for a possible variability between different samples. Typically, reaction mixture is incubated for 90 min at 37 °C, after which the reaction is quenched via addition of cold SAM to a final concentration of 120 mM. Samples are frozen on dry ice and stored at – 20 °C until further analysis.

In order to quantify the amount of total radioactivity incorporated into the product, RNA is purified from the rest of the reaction mixture. We commonly separate the RNA by using the RNA Clean & Concentrate kit (Zymo Research), a method that we find more consistent than phenol/chloroform extractions. Following purification, RNA is eluted in 100 μ l of water and added directly into a scintillation vial containing 5 ml of Ultima Gold Scintillation Cocktail (Perkin-Elmer). Samples are mixed by vigorously shaking and left to rest in the dark overnight. Each scintillation vial is then counted on LSC (Beckman Coulter LS6500 multipurpose scintillation counter) for 5 min.

5.4 Protocol for Determining the Regiochemistry of Methylation by RlmN and Cfr

In order to establish the regioselectivity of the modification on the adenosine ring by RlmN and Cfr, the radiolabeled RNA from the *in vitro* assay is purified and enzymatically digested to single nucleosides. The resulting nucleosides are analyzed by HPLC, and their retention times compared to synthetic standards of predicted methylated products, 2-methyladenosine (RlmN), or 8-methyladenosine and 2,8-dimethyladenosine (Cfr).

Following the purification of the product RNA as described above (Section 5.3), RNA is enzymatically digested using nuclease P1 (Sigma-Aldrich), snake venom phosphodiesterase (Sigma-Aldrich), and Antarctic phosphatase (New England BioLabs). First, 1/20th volume of 1 M NH_4OAc pH 5.3 is added to purified rRNA, followed by nuclease P1 (4 U/50 μ g of RNA) and incubated at 45 °C for 3 h. 1/40th volume of 1 M NH_4HCO_3 and snake venom phosphodiesterase (4 mU/50 μ g of RNA) are then added and incubation is continued for an additional 2 h at 37 °C. Lastly, 1/10th volume of 10 $\times$ Antarctic phosphatase reaction buffer (500 mM bis-Tris-propane HCl pH 6, 10 mM MgCl_2 , 1 mM ZnCl_2) is added together with Antarctic phosphatase (1 U/50 μ g of RNA) and the digestion mixture is incubated for 1 h at 37 °C. Upon completion of the reaction, samples are pelleted at 10,000 rpm for 5 min at room temperature. Supernatant is loaded onto a Luna analytical C18 column (10 μ m, 4.6 mm $\times$ 250 mm), in a solvent system consisting of 40 mM ammonium acetate, pH 6.0 (A) and 40% aqueous acetonitrile (B). The nucleotides are eluted of the column at a flow rate of 1 ml/min with a step gradient of 0% B (0–2 min), 0–25% B (2–27 min), and 25–60% B (27–37 min). The retention times for the products are: 8-methyladenosine (29.5 min), 2-methyladenosine (30 min), and 2,8-dimethyladenosine (35 min). The mononucleosides are detected by a Packard radiomatic 515TR flow scintillation analyzer (Perkin-Elmer) and their retention times are compared to the retention times of the synthetic standards monitored by their UV absorption at 254 nm (Fig. 3). Synthetic standards were synthesized in-house following previously published procedures (Van Aerschot et al., 2002; van Tilburg, Gremmen, von Frijtag Drabbe Kunzel, de Groote, & IJzerman, 2003; Yan et al., 2010).

5.5 Protocol for Testing the Activities of RlmN and Cfr Using Nonradioactive SAM

In this procedure, the methylation reaction is performed at a larger scale (typically 200 μ l) in order to assure that sufficient quantities of methylated rRNA product are obtained for the detection by LC/MS analysis. A typical reaction is performed in 200 μ l of the reaction buffer (100 mM HEPES pH 7.5, 100 mM KCl) containing 10 mM MgCl_2 , 200 μ M SAM, 20 μ M RNA, 2 mM DTT, 20 μ M Flv, 2 μ M Flx, 2 mM NADPH, and 20 μ M either RlmN or Cfr. All the reaction components are prepared anaerobic prior to mixing in an anaerobic chamber.

The reaction is initiated by the addition of the enzyme, and incubated at 37 °C for 1 h. Upon completion of the reaction, vials are frozen in liquid nitrogen and stored at – 80 °C. The RNA is then digested to nucleosides via the treatment with nuclease P1, snake venom phosphodiesterase and Antarctic phosphatase as described above (Section 5.4), with the exception that no prior purification of methylated rRNA is performed. Upon completion of the digestion, samples are pelleted at 10,000 rpm for 5 min at room temperature, and filtered through Amicon Ultra (3 kDa MWCO) filters (Millipore). Filters are washed twice with 200 µl of water, and eluent is lyophilized overnight. Nucleosides are then separated by reverse-phase HPLC using the method described above (Section 5.4). Fractions containing 2-methyladenosine, 8-methyladenosine, 2,8-methyladenosine, and 5'-deoxyadenosine are collected separately based on their known retention times, and dried by lyophilization prior to LC/MS analysis. The lyophilized material is then dissolved in water, and analyzed by a Waters Alliance HPLC, equipped with a Waters 248y diode array detector and a Waters/Micromass ZQ single-quadrupole mass spectrometer (Waters). The solutions are loaded onto an Xterra MS C18 column (3.5 µm, 2.1 mm × 50 mm) (Waters) and eluted at a flow rate of 0.2 ml/min with a 6-min gradient from 100% water to 20% acetonitrile. Data are analyzed by MassLynx software version 4.0 (Fig. 4). While this method is less sensitive than radioactivity incorporation, an advantage of this approach is the ability to monitor the coupling between the product formation (methylated adenosines) and the production of 5'-deoxyadenosine formed in the reaction (Fig. 2).

6 *In Vivo* Activity Assay for RlmN and Cfr

6.1 General Overview of the Method

A number of experimental approaches have been used in the past to evaluate RNA modifications in cellular contexts. Some of the commonly used techniques are primer extension analysis, thin layer chromatography of digested RNA, and mass spectrometry (Basturea, 2013; Kellner, Burhenne, & Helm, 2010). Since our goal is to detect the extent of methylation (unmodified, mono-, or dimethylated adenosine) at a specific position, A2503, mass spectrometry of an RNA fragment encompassing this nucleotide is a particularly well-suited method. An alternative approach is a reverse transcriptase primer extension analysis, which has been extensively used to detect different RNA modifications (Boorstein & Craig, 1989; Motorin, Muller, Behm-Ansmant, & Branlant, 2007). However, in comparison to the mass spectrometry method, this method requires additional radioactive labeling. Moreover, reverse transcriptase is sensitive to the secondary structure of RNA (Basturea, 2013; Boorstein & Craig, 1989; Lane, Field, Olsen, & Pace, 1988; Motorin et al., 2007), and does not provide a large difference in the intensity of the stop between mono- and dimethylated A2503. As a result, this method is less well suited for the analysis of RlmN- and Cfr-mediated methylation *in vivo*. Another method commonly used to identify new modifications in RNA is thin-layer chromatography (TLC). In comparison to the mass spectroscopy method, this method requires smaller amounts of purified RNA; however, the harsh chemical conditions can lead to degradation of the labile-modified nucleosides, as well as need for radioactive labeling deterred us from utilizing this approach (Basturea, 2013; Motorin et al., 2007).

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry method is an extensively used analytical approach to assess the post-transcriptional modification status in RNA (Andersen, Porse, & Kirpekar, 2004; Douthwaite & Kirpekar, 2007; Kirpekar, Douthwaite, & Roepstorff, 2000). The advantages of MALDI-TOF MS technique include: sensitivity, tolerance to impurities, the ability to handle complex mixtures without any prior fractionation, and relative simple sample preparation (Douthwaite & Kirpekar, 2007). Moreover, MALDI spectra are easy to interpret since they are dominated by single charged ions. Although amenable to analysis of RNAs as long as intact rRNAs (Berhane & Limbach, 2003), in most protocols, RNA is digested with an appropriate RNase (e.g., RNase T1, RNase A, RNase H, or RNase U) prior to MS analysis in order to obtain fragments that are less than 20 nucleotides long.

The activity of an RNA modifying enzyme can be identified *in vivo* by comparing the modified nucleotides in RNAs isolated from the wild-type strain to those of a knockout strain that does not contain the gene encoding for the methylating enzyme. Moreover, it is feasible to test the activity of a putative RNA modifying enzyme from various species using *E. coli* or *S. cerevisiae* as a host. In this approach, the putative methylase gene is cloned into a plasmid suitable for transformation into a bacterial or a yeast strain lacking the homologous methylating enzyme to ensure that the methylation site is not premodified (Fig. 1). Our protocol for analysis of RlmN and Cfr activity *in vivo* is based on a method published by Anderson and coworkers, with several modifications (Andersen et al., 2004). The enzyme of interest is expressed in cells during the mid-log phase, and the total RNA isolated prior to the culture reaching the stationary phase. In order to detect both the RlmN-like C2 methylation and Cfr-like C8 methylation of A2503, both the WT *E. coli* and the BW2113/ *rlmN* are used as host strains. In the WT strain, the C2 position of A2503 in 23S rRNA is already premodified by the endogenous copy of *rlmN*, preventing its use as a substrate of transformed RlmN-like enzymes. On the other hand, A2503 in BW2113/ *rlmN* strain is unmodified and can be used to evaluate RlmN-like methylation. Since the C8 position of A2503 is unmodified in both strains, both can be used to evaluate Cfr-like activity.

6.2 Protocol for Isolation of Total rRNA from Strains Overexpressing RlmN and Cfr

E. coli BL21(DE3) and BW2113/ *rlmN* strains transformed with a plasmid encoded RlmN or Cfr (e.g., pET21a-RlmN, pET15b-Cfr, pZA-RlmN(Cfr)) are grown in the LB medium at 37 °C and 180 rpm to an OD₆₀₀ of 0.1. At this point, production of the protein is induced by the addition of either IPTG (for pET vector—final concentration of 200 μM) or anhydrous tetracycline (ATH; for pZA vector—final concentration 20 ng/ml), and growth of the culture is continued for an additional 3.5 h at 18 °C. Following incubation, the cells are harvested by centrifugation (10 min, 4 °C 3000 rpm). Ten milliliters of the culture is sufficient for isolation of enough RNA for the subsequent analysis. The cell pellets are thawed on ice, and then resuspended in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). Lysozyme is added to a final concentration of 2 mg/ml, and the resuspensions are left at room temperature for 10 min. The total RNA is purified using the RNeasy Midi Kit (Qiagen) following manufacturer's recommendations.

6.3 Protocol for Isolation of a Defined rRNA Fragment

To isolate a region of 23S rRNA that contains A2503, 2000 pmol of a synthetic oligodeoxynucleotide complementary to C2480–C2520 nucleotide region of *E. coli* 23S rRNA is incubated with 200 pmol of total RNA in 0.3 volumes of hybridization buffer (250 mM HEPES pH 7, 500 mM KCl). The hybridization mixture is incubated at 90 °C for 7 min, and subsequently slowly cooled to 45 °C over 3.5 h in order to allow for hybridization to occur. Single-stranded RNA and DNA are digested with mung bean nuclease (30 units, New England BioLabs) and RNaseA (0.5 µg, Sigma–Aldrich) over 1 h at 37 °C in a buffer solution (50 mM NaOAc pH 5, 30 mM NaCl, and 1 mM ZnCl₂). The RNA:DNA hybrid is extracted by the addition of 1 volume of a 1:1 phenol–chloroform solution, and the hybrid is precipitated from the aqueous layer by addition of 3 M NaOAc pH 5 (1/10th volume) and ethanol (3 volumes). Following an overnight precipitation at -20 °C, the precipitate is dissolved in a 1:2 solution of H₂O:formamide. The RNA:DNA hybrid is separated by gel electrophoresis (13% polyacrylamide gel containing 7 M urea, 80 V), and bands are visualized by ethidium bromide staining. The RNA band is excised and the RNA is eluted overnight at 4 °C in 200 µl of 1 M NH₄OAc pH 5.3. The RNA is subsequently precipitated overnight at -20 °C with 2 volumes of a 1:1 mixture of ethanol and isopropanol. The precipitate is pelleted by centrifugation at 14,000 rpm for 40 min at 4 °C. The supernatant is then removed and the pellet washed with 70% aqueous EtOH to completely remove any residual SDS and free salts. Subsequently, the RNA is pelleted, solvent is removed, and sample is left to air dry.

6.4 Protocol for RNase Digestion and MALDI-TOF Analysis

The isolated RNA fragment (Section 6.3) is first dissolved in nuclease-free water (typically 10–15 µl) and the sample concentration is determined by NanoDrop. A solution containing 25–30 pmol of isolated RNA is mixed with 0.5 volumes of freshly prepared 0.5 M 3-hydroxypicolinic acid (3-HPA). The RNA is then digested with 500 units of RNase T1 (USBiological) for 1 h at 37 °C. Cyclic phosphates that result from the RNase T1 digestion are linearized by the addition of 0.25 volume of 0.5 M HCl (room temperature, 30 min). The samples are lyophilized, and then redissolved in 5 µl of water. One microliter of the sample is spotted onto the target plate and mixed with 1 µl of 0.5 M 3-HPA matrix. The sample is allowed to dry, and spectra recorded in a reflector and positive ion mode on a Voyager Elite STR MALDI-TOF mass spectrometer (PE-Biosystems). RNase T1 digestion of RNA results in a three nucleotide fragment that encompasses A2503. The *m/z* ratio of the monomethylated fragment is 1013.16, while *m/z* ratio of the dimethylated fragment is 1027.18. A representative MALDI spectrum of both mono and dimethylated fragment is shown in Fig. 5.

7 Summary and Concluding Remarks

While RNA methylation occurs in all kingdoms of life, the type and the distribution of different methylated species varies substantially among archaea, bacteria, and eukaryotes. The most prevalent type of RNA methylation is methylation of nucleobases. However, despite recent advances in our knowledge of these marks, the biological roles of such modifications are still incompletely understood (Machnicka et al., 2013; Motorin & Helm,

2011; Sergeeva et al., 2014; Sergiev et al., 2011). A number of mechanisms have evolved to enable RNA methylation, which are tuned to the electronic demands of the substrate. Herein, we provide an overview of methods for expression, purification, and activity analysis of a specific type of RNA methylating enzymes, radical SAM methylsynthases. These enzymes modify the amidine carbon atoms of an adenosine, A2503, in bacterial 23S rRNA. The activities of these enzymes have only been recently reconstituted (Yan et al., 2010), which can be attributed to the complex anaerobic catalysis that they perform. As the substrate A2503 is located at the nascent peptide exit tunnel of the bacterial ribosome, methylations catalyzed by these enzymes have profound impact on the biology of the host strain. RlmN, an endogenous protein found in all bacteria, methylates the C2 amidine carbon and contributes to the translational fidelity (Benitez-Paez et al., 2012; Ramu et al., 2011; Vazquez-Laslop, Ramu, Klepacki, Kannan, & Mankin, 2010). Cfr, found in pathogenic species, methylates the C8 amidine carbon, a modification that confers resistance to various classes of antibiotics (Giessing et al., 2009; Long et al., 2006; Smith & Mankin, 2008). Interestingly, C2 methylated adenosine was recently detected in a subset of tRNAs, raising the question of the physiological role of this modification (Benitez-Paez et al., 2012). With an increase in available whole genome sequences, the development of methods to identify target substrates of RNA methylating enzymes (Khoddami & Cairns, 2013; Meyer et al., 2012; Tim, Katharina, & Matthias, 2010), as well as advances in the characterization of their activities, we anticipate the coming years will unravel novel aspects of mechanisms of the RNA methylation and deepen insight into the function of the resulting modification.

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Figure 1. Outline of *in vivo* and *in vitro* strategies used to characterize methylation status of specific nucleotide in 23S rRNA by radical SAM methylating enzymes RlmN and Cfr.

Figure 2.
Proposed mechanism of RNA methylation by the radical SAM enzymes RlmN and Cfr.

Figure 3.

HPLC analysis of the methylation products from RlmN and Cfr reactions. (A) Reaction of RlmN with 23S rRNA isolated from BW2113/ *rlmN* strain; (B) reaction of Cfr with 23S rRNA isolated from BW2113/ *rlmN* strain. (a) Reverse-phase HPLC flow ^3H radiogram of digested RNA; (b) reverse phase HPLC UV-vis chromatogram of digested RNA measured at 254 nm; and (c) reverse phase HPLC UV-vis chromatogram of synthetic methyladenosine standards at 254 nm. (1) m^8A ; (2) m^2A ; and (3) $\text{m}^2\text{m}^8\text{A}$.

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Figure 4. Liquid chromatography/mass spectrometry (LC/MS) analysis of methylated adenosine isolated from the reaction of RlmN with rRNA (2496–2582). This reaction was performed with cold SAM.

Figure 5.

MALDI-TOF analysis of Cfr methylation *in vivo*. Fragment C2480–C2520 of *E. coli* 23S rRNA, isolated from BW21113/ *rlmN* strain transformed with pZA-Cfr plasmid, was digested with RNase T1 and fragments were analyzed by MALDI-TOF. Masses of the expected fragments from RNase T1 digestion, based on known nucleotide modifications, are shown in the inset. There is a slight shift in observed m/z values in comparison to predicted values due to difference in calibration of the instrument. m²A is 2-methyladenosine, m⁸A is 8-methyladenosine, and Ψ is pseudouridine.