doi:10.1016/j.jmb.2008.03.024 J. Mol. Biol. (2008) 378, 969–975

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

COMMUNICATION

Interaction of the Tylosin-resistance Methyltransferase RlmA^{II} at its rRNA Target Differs from the Orthologue RlmA^{I}

Stephen Douthwaite[1]*, Lene Jakobsen[1], Satoko Yoshizawa[2] and Dominique Fourmy[2]

[1]*Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark*

[2]*Laboratoire de Chimie et Biologie Structurales, ICSN-CNRS 1 ave de la terrasse, 91190 Gif-sur-Yvette, France*

Received 18 February 2008; received in revised form 11 March 2008; accepted 13 March 2008
Available online 19 March 2008

Edited by D. E. Draper

RlmA^{II} methylates the N1-position of nucleotide G748 in hairpin 35 of 23 S rRNA. The resultant methyl group extends into the peptide channel of the 50 S ribosomal subunit and confers resistance to tylosin and other mycinosylated macrolide antibiotics. Methylation at G748 occurs in several groups of Gram-positive bacteria, including the tylosin-producer *Streptomyces fradiae* and the pathogen *Streptococcus pneumoniae*. Recombinant *S. pneumoniae* RlmA^{II} was purified and shown to retain its activity and specificity *in vitro* when tested on unmethylated 23 S rRNA substrates. RlmA^{II} makes multiple footprint contacts with nucleotides in stem–loops 33, 34 and 35, and does not interact elsewhere in the rRNA. Binding of RlmA^{II} to the rRNA is dependent on the cofactor *S*-adenosylmethionine (or *S*-adenosylhomocysteine). RlmA^{II} interacts with the same rRNA region as the orthologous enzyme RlmA^{I} that methylates at nucleotide G745. Differences in nucleotide contacts within hairpin 35 indicate how the two methyltransferases recognize their distinct targets.

Keywords: rRNA structure; protein–RNA interaction; chemical footprinting; methyltransferases; drug resistance

The activity of ribosomal RNA is fine-tuned by the addition of nucleotide modifications post-transcriptionally. Nucleotide modifications modulate the interaction of rRNA with r-proteins during subunit assembly; they facilitate subunit association to form translationally active ribosomes; and they confer resistance to numerous ribosome-targeted antibiotics.[1–3] In bacterial rRNAs, the majority of modifications are methylations at the nucleobase or the 2′-O of the ribose. Generally, addition of each of these modifications requires a specific methyltransferase enzyme acting together with the cofactor *S*-adenosyl-l-methionine (SAM) as the donor of the methyl group.[4] The methyltransferase RlmA^{II} (formerly termed TlrB) transfers a single methyl group from SAM to the N1 position of nucleotide G748, situated within the loop of 23 S rRNA hairpin 35.[5]

RlmA^{II} homologues are found in several groups of Gram-positive bacteria, notably including *Streptomyces fradiae*, the producer of the macrolide antibiotic tylosin.[6–8] Methylation of G748 confers resistance to tylosin and closely related antibiotics by a synergistic mechanism involving monomethylation at nucleotide A2058.[9] In the crystal structures of ribosomal particles,[10–14] nucleotides G748 and A2058 are about 15 Å apart on opposites faces of the peptide tunnel at the binding site of macrolide, lincosamide and streptogramin B (MLS$_B$) antibiotics,[15,16] and tylosin spans this region of the tunnel to contact both G748 and A2058.[17]

We have developed a model system using the RlmA^{II} from the Gram-positive pathogen *Streptococcus pneumoniae*.[18] The streptococcal methyltransferase recognizes its rRNA substrate in a manner similar to that of other RlmA^{II} homologues and methylates nucleotide G748 before assembly of the

**Corresponding author.* E-mail address: srd@bmb.sdu.dk.

Abbreviations used: DMS, dimethyl sulphate; MLS$_B$, macrolide, lincosamide and streptogramin B; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

23 S rRNA into the 50 S particle.[8] The solution structure of a 24 nt transcript of hairpin 35 was determined by NMR spectroscopy,[18] and an analysis of its interaction with RlmA^{II} showed that a large proportion of the hairpin loop including the G748 target interacts with the methyltransferase. Despite making the extensive loop interaction, RlmA^{II} methylated the hairpin 35 transcript much less efficiently than an authentic 23 S rRNA substrate. A more recent study revealed that effective binding and methylation by RlmA^{II} requires additional elements in rRNA helices 33 and 34 as well as the three-way junction linking these structures to hairpin 35 (Fig. 1).[19]

RNA recognition by RlmA^{II} appears to be similar to that of the orthologous methyltransferase, RlmA^{I} (formerly RrmA) that is found in several groups of Gram-negative bacteria. RlmA^{I} and RlmA^{II} have a common evolutionary origin that is reflected in their degree of amino acid sequence identity (~30%).[20] RlmA^{I} also interacts with helices 33, 34 and 35, and methylates the rRNA before 50 S subunit assembly.[21] An important distinction, however, is that RlmA^{I} methylates the neighbouring guanosine nucleotide at position 745.[22] The sequence of hairpin 35 loop and particularly guanosine at positions 745 and 748 are highly conserved in all bacteria.[23] However, even with these similarities in the rRNA substrates and in

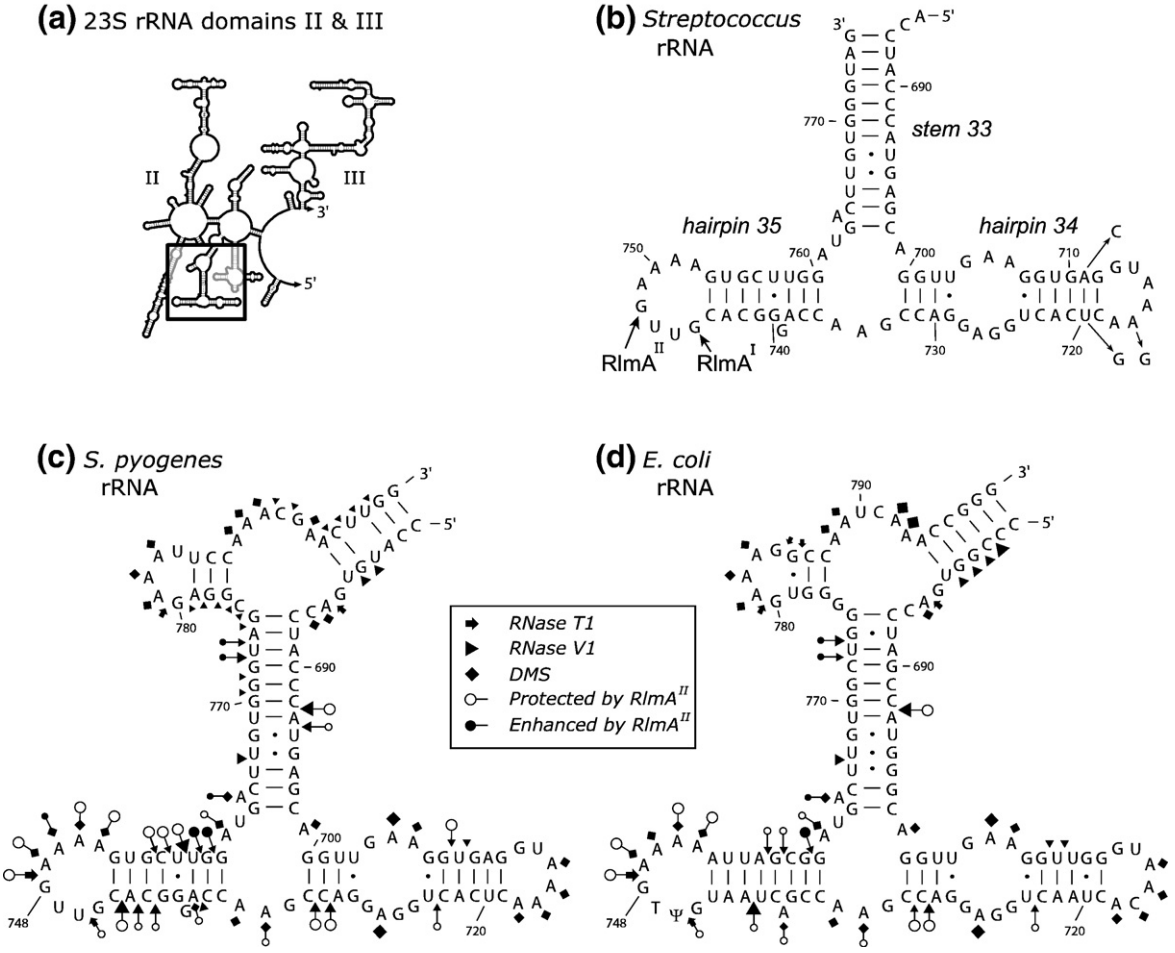

Fig. 1. (a) A representation of the secondary structure within domains II and III of bacterial 23 S rRNA showing the RlmA methyltransferase binding site (boxed).[23,27] (b) Detailed view of the same region of *Streptococcus pyogenes* 23 S rRNA with the methylation targets for RlmA^{I} (G745) and RlmA^{II} (G748). The sequence of this region of the *S. pyogenes* rRNA differs in *Streptococcus pneumoniae* only at the three positions shown at the end of hairpin 34. (c) Nucleotides in the *S. pyogenes* rRNA and in (d) *Escherichia coli* rRNA that are accessible to the T1 and V1 ribonucleases and dimethylsulphate (DMS) probes are shown by the symbols (see the box). A high degree of nucleotide accessibility is indicated by the larger symbols. Open and filled circles indicate nucleotides that are protected or made more accessible, respectively, by binding the RlmA^{II} methyltransferase with the SAH cofactor (the larger the circle, the larger the effect). RlmA^{II} preparation: the recombinant methyltransferase was expressed as a glutathione *S*-transferase fusion protein, and was purified and concentrated as described.[18] Briefly, the fusion protein was cleaved by incubation overnight at 25 °C with thrombin (Sigma) in elution buffer containing 150 mM NaCl. After dialysis, the mixture was loaded onto a DEAE Sepharose column (Pharmacia) equilibrated in 20 mM Tris–HCl (pH 7.0), 5 mM DTT, and the protein was eluted with a salt gradient. The protein was dialysed again, and was then applied to a CM Sepharose column (Pharmacia) equilibrated with 10 mM sodium phosphate (pH 6.4), 5 mM DTT. RlmA^{II} was eluted with a salt gradient and was then dialysed against the phosphate buffer.