Novel One-step Mechanism for tRNA 3′-End Maturation by the Exoribonuclease RNase R of *Mycoplasma genitalium*\(^*\)\

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**Background:** *Mycoplasma genitalium* lacks known ribonucleases for tRNA 3′-processing. The only identified exoribonuclease, RNase R, can carry out this function.

**Results:** RNase R processes the tRNA 3′-end depending on the acceptor stem, discriminator, and CCA terminus.

**Conclusion:** RNase R can process tRNA by recognizing features within the tRNA.

**Significance:** RNase R may process tRNA 3′-end employing a unique single-step exonucleolytic pathway.

*Mycoplasma genitalium* is expected to metabolize RNA using unique pathways because its minimal genome encodes very few ribonucleases. In this work, we report that the only exoribonuclease identified in *M. genitalium*, RNase R, is able to remove tRNA 3′-trailers and generate mature 3′-ends. Several sequence and structural features of a tRNA precursor determine its precise processing at the 3′-end by RNase R in a purified system. The aminoacyl-acceptor stem plays a major role in stopping RNase R digestion at the mature 3′-end. Disruption of the stem causes partial or complete degradation of the pre-tRNA by RNase R, whereas extension of the stem results in the formation of a product terminating downstream at the new mature 3′-end. In addition, the 3′-terminalCCA sequence and the discriminator residue influence the ability of RNase R to stop at the mature 3′-end. RNase R-mediated generation of the mature 3′-end prefers a sequence of RCCN at the 3′-terminus of tRNA. Variations of this sequence may cause RNase R to trim further and remove terminal CA residues from the mature 3′-end. Therefore, *M. genitalium* RNase R can precisely remove the 3′-trailer of a tRNA precursor by recognizing features in the terminal domains of tRNA, a process requiring multiple RNases in most bacteria.

In essentially all organisms, tRNA species are made from primary transcripts containing extra sequences. These extra sequences are removed by nucleolytic processing activities. The 5′-leader sequences in tRNA precursors are removed ubiquitously by RNase P. At the 3′-end, the extra sequences are removed by a variety of different mechanisms. In bacteria, tRNA 3′-processing may be accomplished by the actions of endo- or exoribonucleases or both (1, 2). In *Escherichia coli*, the primary tRNA transcript undergoes an initial cleavage by RNase E in its 3′-trailer downstream of the CCA sequence (3, 4), followed by stepwise trimming reactions of the extra residues by multiple exoribonucleases, including RNases T, PH, D, II, and BN and polynucleotide phosphorylase (5, 6). In *Bacillus subtilis*, pre-tRNAs with an encoded CCA sequence are matured by exonucleolytic action at the 3′-end, whereas CCA-less tRNA precursors are cleaved by RNase Z after the discriminator base, followed by CCA addition (7). In other bacteria such as *Thermotoga maritima*, the 3′-ends of tRNA are matured by a single endoribonucleolytic cleavage after CCA by RNase Z (8). These data demonstrate the existence of diversified mechanisms of tRNA 3′-end maturation in bacteria.

*Mycoplasma genitalium* is the second smallest known bacterium and is considered a model for an organism with a minimal genome. Exhaustive data mining revealed the existence of endoribonucleases RNases P, III, M5, and H3 and a single exoribonuclease, RNase R (9, 10). It is unknown how this organism carries out RNA metabolism, a process usually requiring the action of numerous RNases in other bacteria. Surprisingly, none of the RNases listed above for tRNA 3′-maturation was identified in *M. genitalium* and related species (2, 9, 10). Recently, we demonstrated that purified RNase R of *M. genitalium* exhibits 3′→5′ exoribonuclease activity that is somewhat different from the activities of its *E. coli* homologues RNases R and II (11). Interestingly, *M. genitalium* RNase R alone is able to remove 3′-trailers efficiently and to generate the mature tRNA 3′-end (11). In contrast, *E. coli* RNase R degrades pre-tRNA and other structural RNAs (11–13), whereas *E. coli* RNase II generates mature tRNA poorly (5, 11). These observations suggest that in the presence of a limited number of RNases, *M. genitalium* RNase R may have acquired a unique exonucleolytic tRNA 3′-processing function.

To carry out tRNA 3′-maturation, *M. genitalium* RNase R must be able to recognize pre-tRNA and precisely remove the trailer sequences to form the mature 3′-end. Several sequence and structural features of tRNA have been previously shown to be important for tRNA 3′-processing by other enzymes. For instance, the *E. coli* tRNA 3′-maturation exoribonuclease RNase T is strongly inhibited by C residues in the 3′-CCA sequence, suggesting a role for these C residues in stopping
digestion of tRNA by this enzyme once the mature 3′-end is generated (14). B. subtilis RNase Z cleaves CCA-less tRNA precursors after the discriminator base. However, its activity is inhibited if a C residue is present immediately downstream of the discriminator base (7). In addition, it was found that the terminal double-stranded stem structure present in the precursors of many stable RNA species is essential for exonucleolytic processing to stop correctly at the mature 3′-end (15, 16).

In this work, we report that M. genitalium RNase R demonstrates novel specificity for the nucleotide sequence and structure of a pre-tRNA, enabling tRNA 3′-maturation by one-step exonucleolytic removal of a relatively long 3′-trailer. This represents a unique mechanism of tRNA 3′-maturation by which organisms of minimal genome employ only a single exoribonuclease.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligodeoxynucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). [α-32P]UTP was purchased from GE Healthcare. Genomic DNA of M. genitalium G37 was obtained from American Type Culture Collection (Manassas, VA). *Taq* DNA polymerase was the product of 5 PRIME. T7 RNA polymerase and RNase inhibitor were obtained from New England Biolabs. RNA 5′-polyphosphatase was from Epicentre Biotechnologies (Madison, WI). The plasmid pET15b harboring the gene encoding M. genitalium RNase R (pETmgR) and the *E. coli* expression strain Rosetta-gami 2(DE3)pLysS were described previously (11). SequaGel for denaturing urea-PAGE was the product of National Diagnostics (Atlanta, GA). All other chemicals and reagents were analytical grade.

**Overexpression and Purification of M. genitalium RNase R**—To prepare *M. genitalium* RNase R free of *E. coli* RNases R and II, a mutant of Rosetta-gami 2(DE3)pLysS devoid of RNases R and II was constructed by sequential P1 transduction of the *rnb*:kan and *mnr*:kan alleles (6, 17). Expression and purification of *M. genitalium* RNase R were carried out as described (11) with minor modifications. Briefly, pETmgR was transformed into Rosetta-gami 2(DE3)pLysS. Transformed cells were grown in LB medium at 37 °C to *A*600 = 0.6, followed by induction using 1 mM isopropyl β-D-thiogalactopyranoside overnight at room temperature. Cells were harvested and processed, and *M. genitalium* RNase R was purified as described (11). Purified RNase R was at least 99% pure based on an SDS-PAGE analysis. The activity of the purified RNase R protein was confirmed by poly(A) degradation assays (11).

**Synthesis of tRNA Precursors**—The PCR product encoding the precursor to tRNA Gly, under the control of a T7 promoter was generated using *Taq* DNA polymerase, *M. genitalium* genomic DNA template, and primers. The pre-tRNA Gly generated by run-off transcription starts at the mature 5′-end of the tRNA and contains a 21-nucleotide (nt) 3′-trailer sequence (5′-ACTTGTGTGTGTCCTCCTCTAT-3′). PCR encoding derivatives of pre-tRNA Gly were generated using mutagenic primers and the DNA for pre-tRNA Gly as the template. The sequences of primers used for constructing various pre-tRNAs are provided in supplemental Table S1. Labeled pre-tRNA Gly were made by *in vitro* transcription using these PCR products, T7 RNA polymerase, and [α-32P]UTP as described previously (11) with the four nucleoside triphosphates at 0.5 mM. *In vitro* transcribed pre-tRNAs were purified using the NEasy® MinElute® cleanup kit (Qiagen, Valencia, CA).

**Preparation of 5′-Monophosphorylated RNA**—*In vitro* transcribed pre-tRNAs containing 5′-triphosphate were treated with RNA 5′-polyphosphatase, which sequentially removes γ- and β-phosphates and produces 5′-monophosphate. Reactions were conducted according to the manufacturer’s protocol. In brief, 5 μg of pre-tRNA transcript was incubated with 30 units of RNA 5′-polyphosphatase and 20 units of RNase inhibitor at 37 °C for 30 min. Treated RNA was purified using NEasy MinElute cleanup kit.

**In Vitro tRNA Processing Reactions by Purified RNase R**—*In vitro* tRNA processing was carried out as described (11). After incubation at 37 °C for 5 or 30 min, the reactions were stopped by the addition of 2 volumes of loading buffer (96% formamide and 1 mM EDTA). The products were separated on an 8% urea-polyacrylamide denaturing gel. The products in the gel were detected using a Molecular Imager™ system (Bio-Rad) and quantified using the accompanying Quantity One® software.

**RESULTS**

*M. genitalium* RNase R Generates a Mature 3′-End from Pre-tRNA Gly Containing a Long 3′-Trailer—The 95-nt pre-tRNA Gly construct starts with triphosphate at the mature 5′-end of the tRNA and contains a 21-nt trailer sequence with an —OH group at the 3′-end. Upon incubation with RNase R purified from *M. genitalium*, a 74-nt product corresponding to the size of the mature tRNA Gly was produced after 5 min and increased at 30 min (Fig. 1A, lanes 2 and 3). This product terminates at the mature 3′-end of tRNA Gly because it comigrated with the 74-nt transcript of the same tRNA (Fig. 1A, lane 7), and the 3′-terminal sequence was previously confirmed by 3′-rapid amplification of cDNA ends (11). In contrast, incubation with buffer alone did not cause any change to the pre-tRNA (supplemental Fig. S1, lanes 1 and 2). Formation of the mature tRNA by RNase R demonstrates that this enzyme alone is able to remove the entire 3′-trailer by an exonucleolytic action.

A 77-nt product was present in small amounts in some pre-tRNA Gly preparations (Fig. 1A, lane 4), presumably due to pre-termination of T7 RNA polymerase. This 77-nt product was also generated by incubation with RNase R, suggesting that it is a true processing intermediate of RNase R. In addition, a 72-nt product corresponding to tRNA lacking the terminal CA residues at the 3′-end was also produced by RNase R (Fig. 1A, lanes 2 and 3), indicating that this exoribonuclease is capable of removing nucleotide residues from the mature 3′-end.

We have also incubated the 74-nt transcript with RNase R. Interestingly, this RNA form was not digested by RNase R even after 30 min of incubation (Fig. 1B, lanes 2 and 3). This result suggests that the 72-nt product from pre-tRNA Gly (Fig. 1A, lanes 2 and 3) was probably generated from a longer 3′-terminus by processive exonucleolytic action of RNase R. Once the tRNA
with a mature 3'‐end is released from RNase R, it may become resistant to further trimming by the enzyme.

tRNA normally contains a 5′‐monophosphate. To examine whether the 5′‐triphosphate present in the in vitro transcript affects 3′‐processing by RNase R, the pre‐tRNA was treated with RNA 5′‐polyphosphatase to generate a 5′‐monophosphate (Fig. 1A, lane 4). Polyphosphatase treatment also produced a major 72‐nt product along with some other minor products, probably due to contamination by a nuclease activity. When the 5′‐monophosphate pre‐tRNA was treated with RNase R, the 77+, 74+, and 72‐nt products were produced (Fig. 1A, lanes 5 and 6) in a manner similar to those from the pre‐tRNA with 5′‐tripophosphate, suggesting that the 5′‐tripophosphate does not have a detectable effect on RNase R‐mediated 3′‐processing. Pre‐tRNA constructs containing 5′‐tripophosphate were used in the subsequent experiments.

**Acceptor Stem Stops RNase R Trimming at 4 Nucleotides downstream of the Double Strand**—Stable bacterial RNA species that undergo 3′‐exonucleolytic processing share a common feature of having a stable double‐stranded stem formed between the 5′ and 3′ termini, followed by 2–4 unpaired nucleotides at the mature 3′‐end (15). This feature supports the notion that stable stems function as “rulers” to stop exonucleolytic trimming at the downstream mature 3′‐ends. Being a processing exonuclease, M. genitalium RNase R may recognize the same terminal stem to stop at the mature 3′‐end of tRNA.

To test this idea, a pre‐tRNA construct (DS+3) containing a 3‐bp extension in the acceptor stem (Fig. 2A) was treated with RNase R. This construct would produce a tRNA of 80 nt if RNase R stops 4 nt downstream of the stem. As shown in Fig. 2B (lanes 5 and 6), the expected 80‐nt RNA and a minor 82‐nt product formed after 5 min of incubation with RNase R and became more abundant after 30 min. Therefore, RNase R stops at similar distances downstream of the acceptor stem of DS+3 and pre‐tRNA Gly. The lack of the 2‐nt shorter product from DS+3 is probably due to a higher stability of the extended stem and the presence of a new adenine discriminator in this construct (see below). Similar behavior was observed previously when a pre‐tRNA containing 2 extra bp in the acceptor stem was treated with RNase R (18).

If the acceptor stem impedes RNase R digestion, disruption of the stem may fail to stop RNase R. This idea is well supported by the results in Fig. 2. Incubation of a 2‐bp disruption construct (DS−2) with RNase R resulted in complete degradation of the pre‐tRNA (Fig. 2B, lanes 8 and 9), demonstrating an essential role for a stable acceptor stem in stopping RNase R at the CCA terminus.

**FIGURE 1. RNase R processes pre‐tRNA Gly but not mature tRNA independent of the 5′‐phosphorylation status.** The RNA substrates were uniformly labeled with 32P and treated with RNase R for the time periods indicated. RNA products were separated and detected as described under “Experimental Procedures.” The sizes of RNA are indicated on the right as length in nucleotides. *A*, the 95‐nt pre‐tRNA Gly constructs containing 5′‐tripophosphate (5′‐ppp) or 5′‐monophosphate (5′ p) and a 21‐nt 3′‐tailer were treated with RNase R. A 74‐nt RNA corresponding to the mature tRNA Gly was included as a size marker. B30 indicates incubation with buffer only for 30 min. B, the 74‐nt RNA transcript corresponding to the tRNA with a mature 3′‐end was treated with RNase R.

A and G Are Preferred Discriminator Bases for RNase R to Stop at the Mature 3′‐End of tRNA—The discriminator base has been recognized as an important determinant for charging tRNA with its cognate amino acid (19) and for maturation of the 5′‐end by RNase P (20). Here, we attempted to investigate the possible role of the discriminator residue in tRNA 3′‐processing by RNase R. Fig. 3 shows the processing products of pre‐tRNA constructs with the four different discriminators. Variations in the discriminator base caused a major change in the ratios of the 74‐ and 72‐nt products and slightly affected the formation of 74‐nt RNA, albeit at a low level (Fig. 2C, lane 10). Apparently, 1‐bp disruptions caused partial degradation and altered the positions where RNase R trimming stopped.

C Residues in the CCA Terminus Play Important Roles in Stopping RNase R at the Mature 3′‐End—Because the 3′‐terminal CCA sequence has been implicated in tRNA 3′‐maturation under a number of circumstances and because the trimming of CCA by RNase R was observed in this work, we studied whether the CCA sequence is a determinant for RNase R‐mediated tRNA processing. As shown in Fig. 4A (lanes 6 and 12), substitution of one or both C residues with A or G increased production of the 72‐nt product. Substitution of the first C had more...
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FIGURE 2. Processing of pre-tRNA^Gly^Gly constructs containing variations in the aminoacyl-acceptor stem. A, diagrammatic representation of pre-tRNA^Gly^Gly and its derivatives containing an extension or a disruption in the acceptor stem. B, processing products of pre-tRNA^Gly^Gly variants containing an acceptor stem extension or a 2-bp disruption by RNase R. C, processing products of pre-tRNA constructs containing a 1-bp disrupted acceptor stem by RNase R. The time of incubation with RNase R is indicated. B indicates incubation with buffer for 30 min. The sizes of RNA products are indicated on the right as length in nucleotides.

FIGURE 3. Processing of pre-tRNA^Gly^Gly constructs containing various discriminator bases. Pre-tRNA^Gly^Gly contains U as the discriminator encoded in the M. genitalium genome. Discriminator variations are indicated at the top. Incubation conditions and RNA sizes were as described in the legends to Figs. 1 and 2. The products were quantified using a PhosphorImager as described under "Experimental Procedures." The percentage of the 74- and 72-nt products of the input pre-tRNA (74+72)/Input was calculated by the sum of radioactivity of the two products divided by the total input radioactivity, normalized by the number of labeled U residues. The ratio of the 74- and 72-nt products (74/72) is also shown. B indicates incubation with buffer for 30 min.

The results in this study demonstrate a delicate mechanism of tRNA 3′-maturation by which the entire 3′-trailer is removed in a single-step exonucleolytic action (Fig. 6). It is likely that RNase R acts processively on tRNA precursors. The precise removal of the 3′-trailer appears to depend on the ability of RNase R to recognize several sequence and structural features of the tRNA. RNase R processing activity is strongly inhibited by the aminoacyl-acceptor stem, stopping mainly 4 nt downstream of the stem to form the mature 3′-end. The discriminator residue and the terminal CCA sequence also play important roles in preventing further trimming of the mature 3′-end by RNase R. These findings suggest that RNase R is able to carry out tRNA 3′-maturation in M. genitalium. However, the results do not rule out the possibility that other factors may also play a role in tRNA 3′-end processing in vivo.

discussion

We have shown that the sole exoribonuclease identified in M. genitalium, RNase R, is able to generate mature tRNA from a transcript that contains a relatively long 3′-trailer sequence. The results in this study demonstrate a delicate mechanism of tRNA 3′-maturation by which the entire 3′-trailer is removed in a single-step exonucleolytic action (Fig. 6). It is likely that RNase R acts processively on tRNA precursors. The precise removal of the 3′-trailer appears to depend on the ability of RNase R to recognize several sequence and structural features of the tRNA. RNase R processing activity is strongly inhibited by the aminoacyl-acceptor stem, stopping mainly 4 nt downstream of the stem to form the mature 3′-end. The discriminator residue and the terminal CCA sequence also play important roles in preventing further trimming of the mature 3′-end by RNase R. These findings suggest that RNase R is able to carry out tRNA 3′-maturation in M. genitalium. However, the results do not rule out the possibility that other factors may also play a role in tRNA 3′-end processing in vivo.
This unique mode of action of RNase R distinguishes this enzyme from exoribonucleases participating in tRNA processing in other bacteria (1, 2, 16). In *E. coli*, for example, the shortening of the long 3'-trailer in a tRNA precursor and the generation of the mature 3'-end are carried out by different RNases. The endoribonuclease RNase E and the processive exoribonucleases RNase II and polynucleotide phosphorylase can efficiently remove the long 3'-trailer, leaving a few extra residues at the 3'-end (3). The last few 3'-extra residues are removed by any of the five exoribonucleases RNases T, PH, D, II, and BN, with T and PH being the most efficient enzymes. RNases T, PH, D, and
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BN are highly specific for 3’-processing of tRNA and other stable RNA species, and they are inactive on most other RNA substrates (16). Importantly, M. genitalium RNase R is able to carry out both shortening of the long 3’-trailer and generation of the mature 3’-end. This represents a novel mechanism for tRNA 3’-maturation that has not been found in other organisms (Fig. 6) (2).

M. genitalium RNase R and its E. coli homologues RNases R and II are members of the RNR exoribonuclease family (9). In contrast to M. genitalium RNase R, E. coli RNase R completely degrades a tRNA precursor, whereas RNase II generates the mature 3’-end of tRNA at very low efficiency (5, 6, 11, 16). Such functional differences may be explained by their different sensitivities to higher order structures in RNA. RNase II degrades single-stranded RNA efficiently but stalls at RNA duplex regions (21). A clamp-like assembly in the RNA-binding domain of RNase II possibly allows single-stranded RNA 3’-ends to enter the catalytic center and blocks double-stranded RNA. During RNA digestion in vitro, RNase II progressively slows down at double-stranded structures, resulting in products that usually contain an average 7–9 nt single-stranded overhang at the 3’-end (22). In contrast to RNase II, E. coli RNase R is able to degrade double-stranded RNA effectively starting from a single-stranded 3’-overhang. E. coli RNase R binds single-stranded RNA tightly within the nuclease domain channel. This helps the separation of the double-stranded RNA region immediately outside of the channel and leads to the resulting single-stranded RNA to the channel for degradation (13). E. coli RNase R also possesses a RNA helicase activity in its CsdA domain that contributes to the degradation of double-stranded RNA (23). M. genitalium RNase R is able to degrade highly structured RNA; however, it is sensitive to the aminoacyl-acceptor stem of tRNA and to RNA 2’-O-methylylation (Ref. 11 and this work). The structural features of M. genitalium RNase R responsible for its selective sensitivity to different RNA structures remain to be elucidated.

M. genitalium RNase R has some interesting properties that are similar to those of E. coli RNase T with respect to tRNA 3’-maturation reactions. First, M. genitalium RNase R stops at the mature 3’-end of tRNA most efficiently when the terminal sequence is RCCN. Alterations of the discriminator and CCA sequences may result in removal of the 3’-terminal CA residues by this enzyme. Second, RNase R appears to be sensitive to C-rich sequences (Fig. 5B), which may help it to stop at the 3’-CCA end. Interestingly, E. coli RNase T is also able to remove part of the terminal CCA sequence in tRNA, demonstrates similar sensitivity to C-rich sequences, and prefers a CCN terminus for 3’-maturation of tRNA (14). RNase T is also able to trim residues that are immediately downstream of a stem structure in stable RNAs (15). However, unlike M. genitalium RNase R, E. coli RNase T does not degrade long 3’-trailer sequences in tRNA precursors (5, 24, 25).

The preference of a purine discriminator for tRNA 3’-maturation seems to be unique for M. genitalium RNase R because this has not been shown for any other exoribonucleases. It should be noted that 31 of 36 M. genitalium tRNAs contain an A or a G discriminator (unpublished observations). RNase R may have evolved to recognize a 3’-terminal RCCN sequence for more efficient processing of most tRNA species in M. genitalium. Recognition of the same sequence has been described for the 5’-end processing enzyme RNase P in E. coli (20). The RNA subunit of RNase P contains a UGG sequence in its P15 loop that forms perfect base pairing with the RCC sequence for correct cleavage at the 5’-end. Strikingly, M. genitalium RNase P also contains the same UGG motif, which presumably recognizes the RCC sequence in its pre-tRNA substrates. Therefore, it is likely that both RNases P and R of M. genitalium have evolved to make use of the same RCCN sequence in pre-tRNA for maturation.

In this work, we observed that pre-tRNAs with altered acceptor stems and 3’-terminal sequences are partially or completely degraded by RNase R. This suggests a possible role for this enzyme in the quality control of tRNA.

In summary, M. genitalium RNase R has a combination of properties found in several other exoribonucleases, making it a unique RNase that may carry out diverse reactions in tRNA processing, RNA degradation, and quality control. This multifunctional enzyme is extremely important for RNA metabolism in M. genitalium because of its limited genome size. It remains to be determined if RNase R has a broad role in RNA processing and degradation in M. genitalium and other related bacteria.

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