Supporting Information

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SI Text

Materials and Methods. ***Abbreviations used.*** RPR, RNase P RNA; RPP, RNase P Protein.

Growth conditions for Methanococcus maripaludis (Mma). *Mma* 900 (Δ*hpt*) was kindly provided by Dr. John Leigh, University of Washington (1). This strain and its derivatives were cultivated in either sealed 5-mL serum tubes or 500-mL serum bottles, as described previously (2, 3), except incubation was at 37 °C and McC medium (a complex medium containing yeast extract, vitamins, and minerals) (4) or McCas medium (McC without yeast extract but with casamino acids) was used instead of a mineral salts medium (1). Hydrogen ($H_2 + CO_2$, 80:20 vol/vol, 3×10^5 Pa) or 100 mM formate (under a gas atmosphere of $N_2 + CO_2$, 80:20 vol/vol, 3×10^5 Pa) was used as the methanogenesis substrate.

Construction of Mma BM100 [Mma 900-$(His)_6$-HA-RPP30 strain]. We used homologous recombination to generate an *Mma* 900 derivative carrying a sequence encoding a tandem $(His)_6$-(HA) tag at the 5′ end of the RPP30 open reading frame (ORF; Table S1) in the chromosome. The underlying principle is to use markerless mutagenesis (1) to replace the native ORF with this affinity-tagged ORF, which was first subcloned into a vector containing both neomycin phosphotransferase (Neo^r, for positive selection) and hypoxanthine phosphotransferase (Hpt, for negative selection with 8-aza-hypoxanthine) genes, and then introduced into *Mma* 900 (Δ*hpt*).

The gene encoding *Mma* RPP30, together with 1 kb of upstream and downstream genomic sequences, was amplified by PCR from *Mma* genomic DNA using appropriate primers (MmaRPP30HR-F and MmaRPP30HR-R; Table S2). The PCR product was digested with *BamH*I and *Kpn*I and cloned into these sites in pBT7 (5) to yield pBT7-*Mma* RPP30 + UD. Subsequently, insertional mutagenesis was used to insert sequences encoding the tags into pBT7-*Mma* RPP30 + UD. Briefly, T4 polynucleotide kinase was used to phosphorylate the primers HH-RPP30-F and HH-RPP30-R (Table S2), whose sequences are juxtaposed in the template but oriented outward. These primers were then used to amplify by PCR the entire pBT7-*Mma* RPP30 + UD sequence and thereby incorporate the $(His)_6$ and HA tags upstream of RPP30 coding sequence. The resulting PCR product was circularized by ligation to generate pBT7-$(His)_6$-HA-RPP30 + UD. The $(His)_6$-HA-RPP30 + UD construct was subcloned from this plasmid into pCRPrtNeo via the *Kpn*I and *BamH*I sites generating pCRPrtNeo-$(His)_6$-HA-RPP30 + UD.

Mma 900 (Δ*hpt*) was transformed with 1 μg of pCRPrtNeo-$(His)_6$-HA-RPP30 + UD using the polyethylene glycol (PEG) method (6). The transformants were grown overnight in McC liquid medium under $H_2 + CO_2$ without shaking (7). One hundred μL of this culture were then transferred to 5-mL McC medium containing 1 mg/mL neomycin sulfate. After overnight growth, 100 μL of this culture were plated onto a McC agar plate containing 0.5 mg/mL neomycin sulfate and incubated in an anaerobic jar pressurized with $H_2 + CO_2 + H_2S$ (79.9:20:0.1 vol/vol) to 2×10^5 Pa. Colonies of merodiploid strains formed after 4–7 days of incubation. To allow accumulation of segregants arising from recombinations between upstream or downstream elements, merodiploid cells from one colony were transferred to neomycin-free McCas medium, and the culture was grown overnight under $H_2 + CO_2$. One hundred μl of this culture were plated onto a McCas plate containing 250 μg/mL of 8-aza-hypoxanthine as a counterselective agent to obtain colonies of segregants (tagged and wild-type strains); the gas atmosphere was $H_2 + CO_2$ (80:20, vol/vol) with H_2S (1,000 ppm). Using the primers Ver-1F and Ver-2R (Table S2), modification of the RPP30 chromosomal locus was confirmed by PCR-based amplification of the expected 785-bp product in a segregant representing the tagged strain, which we called *Mma* BM100 [Δ*hpt* $(His)_6$-HA-RPP30]. To validate that *Mma* BM100 did not carry any remnant of the suicide vector backbone, its chromosomal DNA was digested with *Nhe*I and *EcoR*I and analyzed by Southern blotting using pCRPrtNeo-$(His)_6$-HA-RPP30 as the probe. In this analysis, while a single 6.3-kb band was detected for *Mma* 900 consistent with the wild-type locus, two bands (4.9 and 1.5 kb) were observed with *Mma* BM100 as expected for the tagged RPP30 locus.

Purification of native Mma RNase P (wild-type and affinity-tagged variants). Approximately 1 gram of *Mma* 900 cells was resuspended in 10 mL extraction buffer [EB, 20 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM PMSF, and 10% (vol/vol) glycerol] supplemented with 50 mM NaCl, sonicated, and centrifuged at $30,000 \times g$ for 30 min at 4 °C. The supernatant was subjected to ultracentrifugation $100,000 \times g$ for 2 h at 4 °C, resulting in S100, the supernatant expected to have free RNase P, and P100, the ribosomal pellet. Activity assays revealed that P100 also contained RNase P activity. Therefore, to recover the ribosome-associated RNase P, P100 was resuspended in EB500 (EB supplemented with 500 mM NaCl) by gentle agitation for 2 h at 4 °C; a similar approach was used for isolating the bacterial RPP (8). *Mma* RNase P, dissociated from the ribosome, was recovered by ultracentrifugation at $100,000 \times g$. This new supernatant, which we termed S100*, was combined with S100, and the pool was subjected to sequential purification with ion-exchange chromatography columns (5-mL HiTrap heparin- and Q-Sepharose, GE Healthcare). All the inputs were dialyzed in EB50 before loading on columns and a linear NaCl gradient (50–2,000 mM) was used to elute RNase P. Typically, *Mma* RNase P eluted between ~600 and 800 mM NaCl from these matrices.

In addition, *Mma* BM100 was also used for purification of $(His)_6$-HA-RPP30-associated native *Mma* RNase P. We followed the same purification scheme as the untagged, native *Mma* RNase P except that the Q-Sepharose step was replaced by immobilized metal affinity chromatography (IMAC) using a 1-mL HiTrap chelating column precharged with Ni^{2+}. When this affinity column is subjected to a linear 0- to 500-mM imidazole gradient, $(His)_6$-HA-RPP30-associated *Mma* RNase P typically elutes around 150 mM imidazole.

Western analysis. The partially purified fractions corresponding to the peak of RNase P activity (from either Q-Sepharose or IMAC) were subjected to trichloroacetic acid precipitation. Samples were then separated using tricine-SDS-PAGE (9) and transferred to Hybond™-ECL membrane (GE Healthcare) for Western blot analysis. The primary polyclonal antiserum was generated by immunization of a rabbit with purified recombinant *Mma* L7Ae (28-day Quick Draw Protocol, Pocono Rabbit Farm and Laboratory Inc.); the serum was diluted 1:500 prior to use. We used ECL Plex goat anti-rabbit IgG, Cy5 (GE Healthcare) as the secondary antibody (2,500-fold dilution). Imaging was performed at 700 nm using the Odyssey infrared imaging system (LI-COR Biosciences).

Mapping the 5′ and 3′ ends of Mma RNase P RNA (RPR). We used 5′ and 3′ RACE (rapid amplification of cDNA ends) to map the terminal sequences of the native *Mma* RPR. First, total RNA was extracted from *Mma* cells using TRIZOL® reagent (Invitrogen). To map the 5′ end, a reverse primer specific to RPR (MmaRPR-R, Table S2) and ThermoScript™ reverse transcriptase (Invitrogen) were used to generate the RPR's cDNA from total RNA. The cDNA-RNA hybrid was treated with RNase H to digest the RPR template. Next, the cDNA was treated with terminal transferase and dATP to generate a poly-A tail at its 3′ end. This A-tailed cDNA was amplified by PCR using as primers oligo-dT (20-mer) and MmaRPR-R (Table S2). The product was cloned into pBT7 (linearized with *Stu*I, blunt-end ligation) and sequenced. To map the 3′ end, total RNA was treated with poly-A polymerase and rATP to generate a poly-A tail at the 3′ end of the RNAs. Next, cDNAs were generated using the oligo-dT primer, and the RPR sequence amplified using oligo-dT and an RPR-specific forward primer (MmaRPR-F; Table S2). The PCR product was cloned into pBT7 and sequenced.

The full-length *Mma* RPR sequence (as determined by our RACE studies) is shown in Fig. S1. In comparison, the one used in our in vitro reconstitution studies lacks 4 and 5 nts at the 5′ and 3′ termini, respectively. Because we found that the presence of these extensions reduces activity by 20% under conditions optimal for the shorter variant and we had already invested significant in vitro reconstitution efforts with it, we proceeded to complete the entire study with the shorter (more active) derivative.

Cloning and mutagenesis of Mma RPR. The gene encoding *Mma* RPR was amplified by PCR using *Mma* genomic DNA as the template and MmaRPR-F and MmaRPR-R as the gene-specific primers (Table S2). The PCR product was then digested with *Eco*RI and cloned into pBT7 that had been digested with *Stu*I and *Eco*RI. The resulting plasmid, pBT7-*Mma* RPR^{wt} has the *Mma* RPR gene placed under the control of a T7 RNA polymerase promoter.

RPR mutants were generated by PCR-based site-directed mutagenesis with pBT7-*Mma* RPR^{wt} as the template and the desired mutations incorporated in two primers whose sequences were designed to abut each other in the template but face outward (Table S2). In brief, T4 polynucleotide kinase was used to phosphorylate the two primers, which were employed to amplify by PCR the entire pBT7-*Mma* RPR^{wt} plasmid sequence and thereby incorporate the desired changes in the *Mma* RPR coding sequence. The different RPR mutants were made using the following primer pairs: Ia, MmaRPR-mutA → C-FIa and MmaRPR-mutA → C-RIa; IIa, MmaRPR-mutA → C-Flla and MmaRPR-mutA → C-RIIa; Ib, MmaRPR-mutU → C-F and MmaRPR-mutU → C-RIb; and IIb, MmaRPR-mutU → C-F and MmaRPR-mutU → C-RIIb. The resulting PCR products were circularized by ligation and then transformed into *Escherichia coli* DH5α. The transformants were screened to identify the mutant clones, and subsequently sequenced to confirm the presence of the desired mutation.

Generation of Mma RPRs using in vitro transcription. The plasmids carrying either the wild-type or mutant *Mma* RPRs were linearized with *Eco*RI, and used as templates for T7 RNA polymerase-mediated run-off transcription using established protocols (5, 8). The resulting RNA transcripts were dialyzed extensively against double-distilled water and their concentrations determined from Abs_{260} measurements and their respective extinction coefficients.

Cloning and mutagenesis of Mma RPPs. The genes encoding *Mma* RPPs and L7Ae were amplified by PCR with *Mma* genomic DNA as the template and the respective gene-specific primers (Table S2). To overexpress POP5 in *E. coli*, 7 of the first 14 codons were changed to better align with the codon usage preference of *E. coli*. The POP5 and RPP21 PCR products were digested with *BamH*I and cloned into pLANT-2b (10), which was digested with *Nde*I, filled in with Klenow, and then digested with *BamH*I. The RPP29 and RPP30 PCR products were digested with *BamH*I and cloned into pET-15b (Novagen), which was digested either with *Nde*I (for RPP29) or *Nco*I (for RPP30), filled in with Klenow, and then digested with *BamH*I. (*Note*: The RPP29 gene in pET-15b encodes a $(His)_6$ and a thrombin protease cleavage site at the N terminus.) The RPP30 ORF in pET-15b with its upstream T7 promoter was subsequently subcloned downstream of pLANT-2b-*Mma* POP5 using *Bgl*II and *Blp*I to create a tandem *Mma* POP5-RPP30 clone that permits a more even cooverexpression. The PCR product corresponding to the L7Ae ORF was first cloned into pCR®-Blunt (Invitrogen) to generate pCR®-Blunt-*Mma* L7Ae^{wt}, and then subcloned into pET-15b using *Nco*I and *BamH*I. For generating the L7Ae^{N32A,E33A,K36A} mutant, we used pCR®-Blunt-*Mma* L7Ae^{wt} as the template in a PCR-based mutagenesis using as primers MmaL7Ae-mut-F and MmaL7Ae-mut-R (Table S2); subsequently, the *Mma* L7Ae^{N32A,E33A,K36A} ORF was subcloned into pET-15b using *Nco*I and *BamH*I. (*Note*: In all the above instances, PCR products were digested with appropriate restriction enzymes, whose recognition sites were included in the PCR primers.)

Protein overexpression. The plasmids pET-15b-*Mma* RPP29 and pET-15b-*Mma* L7Ae (both wild-type and mutant) were introduced into *E. coli* BL21(DE3) Rosetta cells (Novagen). The transformants were then grown at 37 °C in LB media supplemented with 100 μg/mL carbenicillin and 35 μg/mL chloramphenicol. The plasmids pLANT-2b-*Mma* POP5-RPP30 and pLANT-2b-*Mma* RPP21 were introduced into *E. coli* BL21 (DE3) cells, and the transformants grown in LB media supplemented with 35 μg/mL kanamycin. Induction for all proteins was initiated at Abs_{600} ~0.5 with IPTG. RPP21 and RPP29 were induced with 0.5 mM IPTG for 15 h at 23 °C; for RPP21 overexpression, 1 mM $ZnCl_2$ was also added at the time of induction as the protein requires Zn^{2+} to fold properly (11, 12). L7Ae and the POP5•RPP30 binary complex were induced with 2 mM IPTG for 4 h at 37 °C.

Purification of Mma RPPs.

POP5•RPP30.
The cell pellet from 125 mL of culture was resuspended in 25 mL of buffer A [25 mM Tris-HCl (pH 8), 5 mM DTT, 0.1 mM PMSF, and 1 mM EDTA] supplemented with 1 M NaCl, sonicated, and centrifuged at 12,000 × *g* for 15 min at 4 °C to remove the cell debris. The supernatant was heated at 65 °C for 15 min to denature the majority of *E. coli* proteins and then centrifuged at 12,000 × *g* for 15 min at 4 °C. To precipitate nucleic acids, the supernatant was treated with 0.05% (vol/vol) of polyethyleneimine (PEI), incubated on ice for 20 min, and then centrifuged at 12,000 × *g* for 15 min at 4 °C. Proteins remaining in the supernatant were precipitated using 80% $(NH_4)_2SO_4$ and the pellet containing POP5•RPP30 was resuspended in 20 mL of buffer A, passed through a 0.45-μm filter, and loaded on a 1-mL HiTrap SP-Sepharose column (GE Healthcare). Bound proteins were eluted with a linear 500- to 1,200-mM NaCl gradient, and POP5•RPP30 typically elutes between 700 and 900 mM NaCl.

RPP29.
The cell pellet from 125 mL of culture was resuspended in 25 mL buffer [20 mM Tris-HCl (pH 8), 10 mM imidazole, 500 mM NaCl, and 1 mM EDTA], sonicated, and centrifuged at 12,000 × *g* for 15 min at 4 °C to remove the cell debris. The supernatant was treated with 0.05% (vol/vol) PEI, incubated on ice for 20 min, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant

was passed through a 0.45-μm filter and loaded on a 1-mL HiTrap chelating column (precharged with Ni^{2+}) and eluted using a linear 10- to 500-mM imidazole gradient. The peak fractions (~75–175 mM imidazole) of $(His)_6$-RPP29 were pooled and dialyzed against PBS and then treated with thrombin to remove the N-terminal $(His)_6$ tag. Due to the nature of the cloning in pET-15b, two additional amino acids (Gly and Ser) are present at the N terminus after thrombin cleavage.

RPP21.
The cell pellet from 125 mL of culture was resuspended in 25 mL of buffer A [25 mM Tris-HCl (pH 8), 5 mM DTT, 0.1 mM PMSF, and 1 mM EDTA] supplemented with 1 M NaCl, sonicated, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was treated with 0.05% (vol/vol) of PEI, incubated on ice for 20 min, and centrifuged at 12,000 × *g* for 15 min at 4 °C. Proteins in the supernatant were precipitated using 60% $(NH_4)_2SO_4$, and the pellet containing RPP21 after centrifugation was resuspended in 20 mL of buffer A, passed through a 0.45-μm filter, and loaded on a 1-mL HiTrap heparin-Sepharose column. The peak fractions (1,000–1,400 mM NaCl) were pooled, supplemented with NaCl to 2 M, and then loaded on a 1-mL HiTrap phenyl-Sepharose column. Bound proteins were eluted using a reverse 2,000- to 0-mM NaCl gradient, and RPP21 typically elutes between 800 and 400 mM NaCl.

L7Ae.
Both wild-type and mutant (N32A,E33A,K36A) *Mma* L7Ae were purified using the same method. The cell pellet from 125 mL of culture was resuspended in buffer [100 mM Tris-HCl (pH 9), 5 mM DTT, and 0.1 mM PMSF], sonicated, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was heated at 55 °C for 15 min to denature most of the *E. coli* proteins and then centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was treated with 0.05% (vol/vol) of PEI, incubated on ice for 20 min, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was passed through a 0.45-μm filter and loaded on a 1-mL HiTrap Q-Sepharose column. L7Ae typically elutes between 350 and 400 mM NaCl. The peak fractions of L7Ae were pooled, supplemented with NaCl to 2 M, and loaded on a 1-mL HiTrap phenyl-Sepharose column. Near homogenous L7Ae is found in the flow-through.

Other comments.
An ÄKTA FPLC purifier (GE Healthcare) was used for all chromatographic procedures. SDS-PAGE analysis and Coomassie brilliant blue staining were used to assess the purity of the RPPs in the eluted fractions. The Abs_{280} values for the different *Mma* RPPs were measured and the protein concentrations calculated using their respective extinction coefficients. All purified *Mma* RPPs and L7Ae were dialyzed against assay buffer [50 mM Tris-HCl (pH 8), 500 mM NH_4OAc, and 7.5 mM $MgCl_2$] and supplemented with glycerol to a final concentration of 25% (vol/vol). These protein stocks, typically at a concentration of 50 to 75 μM, when stored at −20 °C were active for at least three months. The proteins were diluted in assay buffer prior to their use in reconstitution assays.

CD spectroscopy. Both *Mma* $L7Ae^{WT}$ and $L7Ae^{N32A,E33A,K36A}$ were dialyzed against 20 mM disodium phosphate (pH 7) and 20 mM NaCl. The CD spectra of these samples (~20 μM) were acquired using a CD spectrophotometer (Aviv Instruments) and a 1-mm path length cuvette. Scans were performed from 195 to 260 nm at 0.1 nm/sec. The final spectrum for each protein represents the average from three independent scans. The raw data were converted to molar ellipticity values based on the molecular weight and the protein concentration of L7Ae.

RNase T1-based footprinting to map Mma RPR-L7Ae interactions. Each 40-μL footprinting reaction contained a trace amount of 5′ [^{32}P]-labeled and 125 nM unlabeled *Mma* RPR^{wt} or $RPR^{GA\rightarrow GC}$ either alone or complex with 1.25 μM of L7Ae, 4 RPPs or 4 RPPs + L7Ae in assay buffer [50 mM Tris-HCl (pH 7.5), 500 mM NH_4OAc, and 7.5 mM $MgCl_2$]. RPR with different RPP combinations was first reconstituted at 37 °C for 5 min before addition of 1 μL of RNase T1 (0.1 U/μL, 10-fold dilution in water, Ambion). The incubations were then continued at 37 °C for either an additional 10 min (RPR alone and RPR + L7Ae) or 20 min (RPR + 4 RPPs and RPR + 4 RPPs + L7Ae). The reactions were extracted with 40 μL of phenol/chloroform and the RNAs precipitated by adding two volumes of ethanol in the presence of 0.3 M potassium acetate (pH 4.8) and glycogen (20 μg/mL). The RNAs were then pelleted at 21,000 × *g* for 15 min and the pellets washed with 70% (vol/vol) ethanol, air dried, and then resuspended in 8 M urea, 0.04% (wt/vol) bromophenol blue, 0.04% (wt/vol) xylene cyanol, 0.8 mM EDTA, 20% (vol/vol) phenol. Approximately 5,000 dpm of each resuspended sample was separated by 10% (wt/vol) polyacrylamide/8 M urea gel electrophoresis and visualized by exposing to an imaging plate and scanning by a Typhoon Trio phosphorimager. Size markers were generated by a partial hydrolysis of the *Mma* RPR in 50 mM sodium bicarbonate (pH 9.2) at 95 °C to obtain an alkaline ladder or with RNase T1 under denaturing conditions [50 mM Tris-HCl (pH 8), 7 M urea, 1 mM EDTA] to obtain a G ladder.

1. Moore BC, Leigh JA (2005) Markerless mutagenesis in Methanococcus maripaludis demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J Bacteriol* 187:972–979.
2. Mukhopadhyay B, Johnson EF, Wolfe RS (1999) Reactor-scale cultivation of the hyperthermophilic methanarchaeon Methanococcus jannaschii to high cell densities. *Appl Environ Microbiol* 65:5059–5065.
3. Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of Methanobacterium ruminantium in a pressureized atmosphere. *Appl Environ Microbiol* 32:781–791.
4. Whitman WB, Shieh J, Sohn S, Caras DS, Premachandran U. (1986) Isolation and characterization of 22 mesophilic methanococci. *Syst Appl Microbiol* 7:235–240.
5. Tsai HY, Lai LB, Gopalan V (2002) A Modified pBluescript-based vector for facile cloning and transcription of RNAs. *Anal Biochem* 303:214–217.
6. Tumbula DL, Makula RA, Whitman WB (1994) Transformation of *Methanococcus maripaludis* and identification of a *Pst* I-like restriction system. *FEMS Microbiol Lett* 121:309–314.
7. Whitman WB, Shieh J, Sohn S, Caras DS, Premachandran U (1986) Isolation and characterization of 22 mesophilic methanococci. *Syst Appl Microbiol* 7:235–240.
8. Vioque A, Arnez J, Altman S (1988) Protein-RNA interactions in the RNase P holoenzyme from *Escherichia coli*. *J Mol Biol* 202:835–848.
9. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379.
10. Finkelstein J, Antony E, Hingorani MM, O'Donnell M (2003) Overproduction and analysis of eukaryotic multiprotein complexes in *Escherichia coli* using a dual-vector strategy. *Anal Biochem* 319:78–87.
11. Amero CD, Boomershine WP, Xu Y, Foster M (2008) Solution structure of *Pyrococcus furiosus* RPP21, a component of the archaeal RNase P holoenzyme, and interactions with its RPP29 protein partner. *Biochemistry* 47:11704–11710.
12. Kakuta Y, Ishimatsu I, Numata T, Kimura K, Yao M, Tanaka I, Kimura M (2005) Crystal structure of a ribonuclease P protein Ph1601p from *Pyrococcus horikoshii* OT3: An archaeal homologue of human nuclear ribonuclease P protein Rpp21. *Biochemistry* 44:12086–12093.

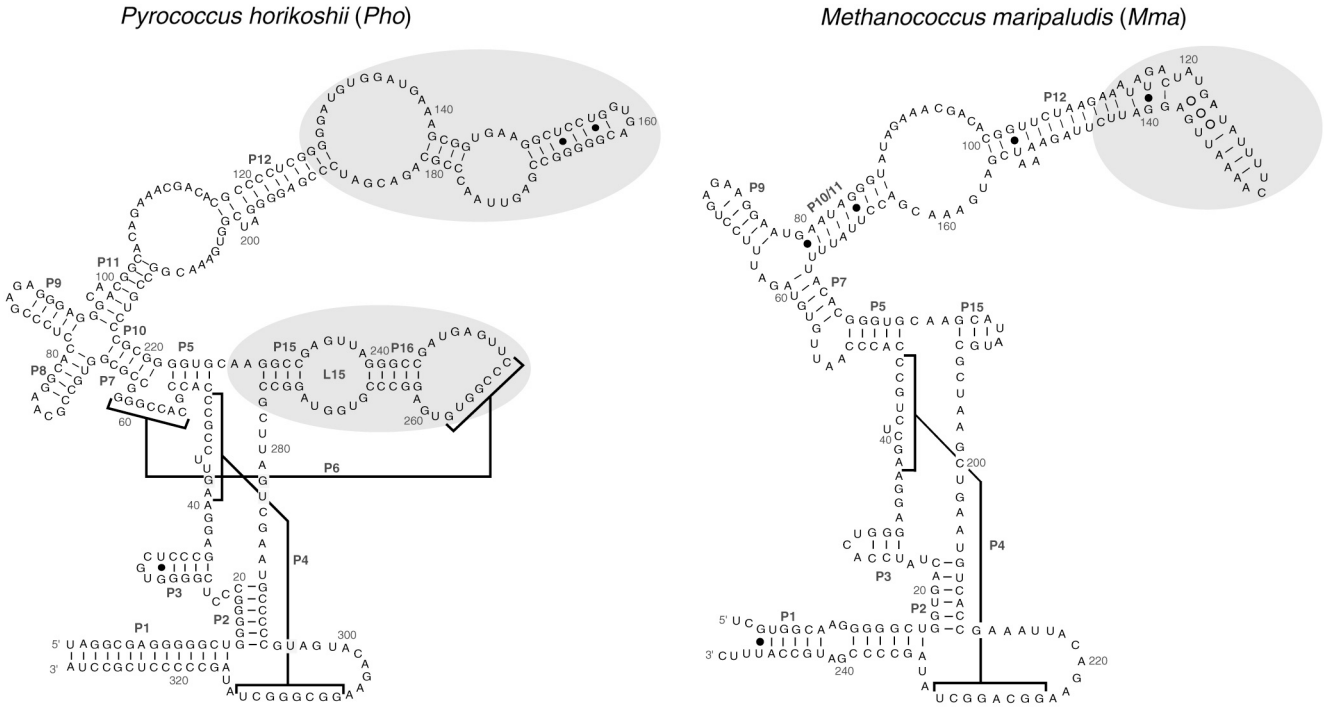

Fig. S1. Secondary structure representations of *Pho* (type A) and *Mma* (type M) RPR. The gray ovals in *Pho* RPR indicate the two possible binding sites for L7Ae identified earlier, whereas the one in *Mma* RPR indicates the region investigated in this study. This figure parallels Fig. 1, except that nucleotide sequences are provided here instead of a cartoon depicting the secondary structure.

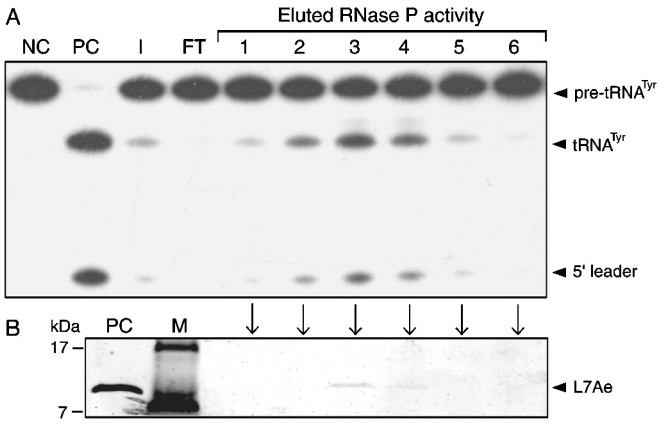

Fig. S2. Coelution of *Mma* RNase P activity and L7Ae from *Mma* BM100 [i.e., *Mma* 900-$(His)_6$-HA-RPP30]. (*A*) Partially purified native *Mma* RNase P (obtained after immobilized metal affinity chromatography) was assayed for pre-tRNA^{Tyr}-processing activity at 37 °C. PC, positive control, generated from processing of pre-tRNA^{Tyr} by in vitro reconstituted *Eco* RNase P. NC, negative control, pre-tRNA^{Tyr} substrate only. I and FT, input and flow-through, respectively. (*B*) Western blot analysis of L7Ae from IMAC fractions of peak activity (*A*) using a rabbit polyclonal antiserum raised against *Mma* L7Ae. PC, positive control, recombinant *Mma* L7Ae. M, size markers.

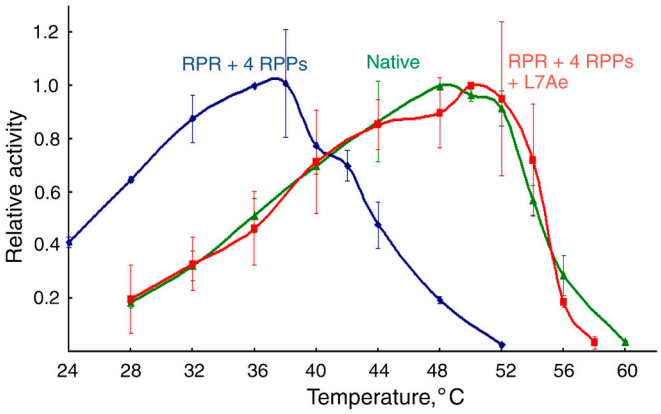

Fig. S3. Comparison of the optimal temperature for pre-tRNA^{Tyr} cleavage by partially purified native *Mma* RNase P (green) and in vitro reconstituted *Mma* RNase P with (red) or without (blue) L7Ae. Although not depicted, the addition of bovine serum albumin had no effect on the profile obtained with the holoenzyme reconstituted with the *Mma* RPR and 4 RPPs. The data presented are the mean of initial velocities calculated from two independent experiments. The highest activity observed (among the various assay temperatures tested) was set as 1.0 and the remainder normalized accordingly to calculate their relative activities.

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Mma     MAVYVKFEISQELEEKTAEVVANAEKIKKGANEVTKAVEKGIAKLVVIAQDVQPEEIVAH 60
Mae     MAVYVKFDVPQEMEEKTAEVLSKSEKVKKGANEVTKAVERGTAKLVVLAKDVQPEEIVAH 60
Mva     MAIYVKFDIPQELEEKTAEVVANAEKIKKGANEVTKAVEKGIAKLVVVAKDVQPEEIVAH 60
Mvo     MAVYVKFEVPQELEEKTAEVVSKATMIKKGANEVTKAVERSRAKLVVVAKDVQPEEIVAH 60
Mja     MAVYVKFKVPEEIQKELLDAVAKAQKIKKGANEVTKAVERGIAKLVIIAEDVKPEEVVAH 60
MFS     MAVYVKFKVPEEIQKELLDAVAKAQKIKKGANEVTKAVERGIAKLVIIAEDVKPEEVVAH 60
Mfe     MAVYVKFKVPEDIQKELLDAVAKAQKIKKGANEVTKAVERGIAKLVIIAEDVQPEEVVAH 60
Min     MAIYVKFKVPEDLQKELLDAVAKAEKIRKGANEVTKAVERKQAKLVIIAEDVKPEEIVAH 60
Mvu     MAVYVKFKVPEEIQKELLDAIAKAQKIKKGANEVTKAVERGIAKLVIIAEDVKPEEVVAH 60

Mma     IPVICDEKGIAYSYSSTKEALGKAAGLEVPTSAIAVVAEGSADELKDLVEKLNGLKA 117
Mae     IPIICEEKGIPYTYIATKEDLGKAIGLEVSTAAVAIIAEKDANALKDLVEKINGLKA 117
Mva     IPVICEEKGIAYSYCSTKEALGKAAGLEVPTSAIAVVAEGSAEQLKDLVEKLNGLKA 117
Mvo     IPAICEEKGIAYTYCATKEDLGKAANLEVPTSAIAIIETEDAEGLNELVEKVNALKA 117
Mja     LPYLCEEKGIPYAYVASKQDLGKAAGLEVAASSVAIINEGDAEELKVLIEKVNVLKQ 117
MFS     LPYLCEEKGIPYAYVASKQDLGKAAGLEVAASSVAIINEGNAEELKALIEKVNALKQ 117
Mfe     LPYLCEEKGIPYAYVASKQDLGKAAGLEVAASSVAIVNEGNADELKALIEKINALKQ 117
Min     LPVLCEEKGIPYAYVASKQDLGKAAGIEVAASSVAIIKPANEEELNALIEKINALKQ 117
Mvu     LPYLCEEKGIPYAYVASKQDLGKAAGLEVATSAVAIVKEGDADELKALIEKINALKE 117
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Fig. S4. Sequence alignment of L7Ae homologs from different Methanococcales. Whereas all conserved residues are highlighted in gray, red boxes indicate conserved amino acids that interact directly with the K-turn (1–3). The NExxK motif was mutated in this study (Fig. 5*A*). Abbreviations not elaborated elsewhere: *Mae*, *Methanococcus aeolicus*; *Mva*, *Methanococcus vannielii*; *Mvo*, *Methanococcus voltae*; *Mja*, *Methanocaldococcus jannaschii*; *MFS*, *Methanocaldococcus* sp. FS406-22; *Mfe*, *Methanocaldococcus fervens*; *Min*, *Methanocaldococcus infernus*; and *Mvu*, *Methanocaldococcus vulcanius*.

1. Hamma T, Ferre-D'Amare AR (2004) Structure of protein L7Ae bound to a K-turn derived from an archaeal box H/ACA sRNA at 1.8 A resolution. *Structure* 12:893–903.
2. Moore T, Zhang Y, Fenley MO, Li H (2004) Molecular basis of box C/D RNA-protein interactions; cocrystal structure of archaeal L7Ae and a box C/D RNA. *Structure* 12:807–818.
3. Vidovic I, Nottrott S, Hartmuth K, Luhrmann R, Ficner R (2000) Crystal structure of the spliceosomal 15.5 kD protein bound to a U4 snRNA fragment. *Mol Cell* 6:1331–1342.

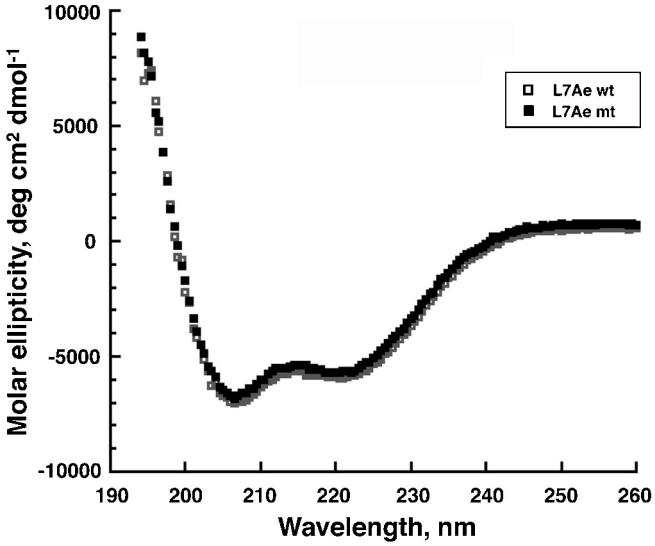

Fig. S5. Circular dichroism spectra of *Mma* L7Ae^{wt} and L7Ae^{N32A,E33A,K36A}.

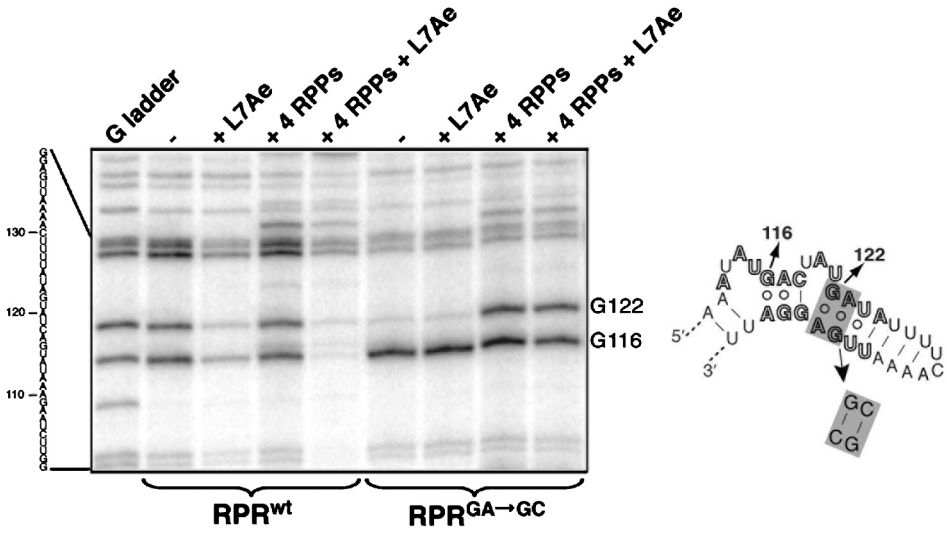

Fig. S6. RNase T1-based footprinting to demonstrate the absence of binding of *Mma* L7Ae to an *Mma* RPR mutant in which the L7Ae binding site (K-turn) is mutated. 5′ end-labeled *Mma* RPR^{wt} and RPR$^{GA→GC}$ mutant were examined either alone (-) or in the presence of L7Ae, 4 RPPs, or 4 RPPs + L7Ae. Molecular size ladders were generated by subjecting end-labeled *Mma* RPR to partial RNase T1 digestion under denaturing conditions. To provide context to the footprinted regions (e.g., G116 and G122), the ladder is annotated and a secondary structure model of the P12 region (Fig. 4*D*) is provided.

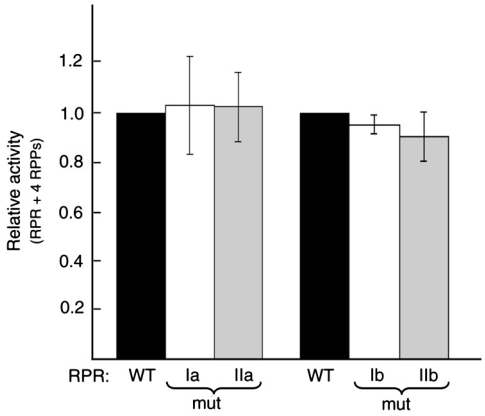

Fig. S7. Comparison of the pre-tRNA^{Tyr}-processing activity of *Mma* RNase P reconstituted with RPR mutant derivatives. The WT or mutant RPRs (Ia, IIa, Ib and IIb; Fig. 4) were tested for activity after reconstitution with the four RPPs. A turnover number of ~5 min^{-1} (at 2,500 nM pre-tRNA^{Tyr}) for the WT holoenzyme was used as the reference to calculate the relative activities of the different mutants. The mean and standard deviation values were calculated from three independent experiments.

Table S1. Characteristics of *Mma* RPPs

RPPs	Gene ID	Isoelectric point	Predicted mass, Da
POP5	MMP0878	10.6	15,149
RPP21	MMP0921	11	12,891
RPP29	MMP1407	10	11,051
RPP30	MMP0430	7.6	26,452
L7Ae	MMP0641	4.6	12,381

Table S2. DNA oligonucleotides used for cloning and mutagenesis of *Mma* RPR and RPPs

MmaRPR-F	CAAGGGGGCTGGTGACTATC
MmaRPR-R	GC***GAATTC***GGGCATCGGGGCTATAGCCTG
MmaRPR-mutA → C-FIa	TTCAAAATTGAGGCTTCTTAGAAAATCGA
MmaRPR-mutA → C-RIa	AATATCATAGGCATATTTCTTAGAACCGTGTCG
MmaRPR-mutA → C-FIIa	TTCAAAATTGCGGATTCTTAGAAAATCGA
MmaRPR-mutA → C-RIIa	AATAGCATAGTCATATTTCTTAGAACCGTGTCG
MmaRPR-mutU → C-F	TTCAAAATTGAGGATTCTTAGAAAATCGA
MmaRPR-mutU → C-RIb	AATATCATAGTCGTATTTCTTAGAACCGTGTCG
MmaRPR-mutU → C-RIIb	AATATCGTAGTCATATTTCTTAGAACCGTGTCG
MmaPOP5-F2	TGCTGAAAACCCTGCCGCCGACCCTGCGTGAAAAAAAACGATACGTCGCA
MmaPOP5-R	AT***GGATCC***TTACTTTTTTGGGCCTTTTCTTTTTAAT
MmaRPP30-F	CTTGAAGGAATTTTTGATATAAATCATGTTT
MmaRPP30-R	AT***GGATCC***TTATTCTTCGACAATTTCGAGTCC
MmaRPP21-F	TAGCTA***CATATG***AAACTAAAAAAGAAATTTCTAGAAAAGTC
MmaRPP21-R	AT***GGATCC***TTATTTTTTTGCCGTTTTTATTGGAATT
MmaRPP29-F	TGTCACAAAATATATTGAGACATGAACTA
MmaRPP29-R	AT***GGATCC***TTAGTACGAGTAGAGCTGTTTTATTTTT
MmaL7Ae-F2	ATCGAT ***CCATGG***CTGTATATGTTAAATTTGAAATATCA
MmaL7Ae-R	AT***GGATCC***TTATGCTTTTAATCCATTTAATTTTTCAAC
MmaL7Ae-mut-F	ACAGCGGCAGTAGAAAAAGGAATTGCAAAATTAGT
MmaL7Ae-mut-R	AACCGCCGCAGCGCCTTTTTTGATTTTTTCAG
MmaRPP30-HR-F	AT***GGTACC***TGCACCAACTGTGTTATCAAC
MmaRPP30-HR-R	AT***GGATCC***CCAGGTACAATCATCCAAAAGAT
HH-Mma30-F	TTA*TACCCATACGACGTTCCAGACTACGCA*CATATGCTTGAAGGAATTTT
HH-Mma30-R	AAC*GTGGTGGTGGTGGTGGTG*GCTAGCCATTAAATCCCCTGAAATTAAAT
Ver-1F	AGGGGATTTAATGGCTAGCC
Ver-2R	TCAGAAAAAAAGATTATTCTTCGAC

Sequences listed above are oriented 5′ to 3′. Restriction sites, if incorporated into primers to facilitate subsequent cloning into appropriate vectors, are shown in bold, italic font. Underlined sequences indicate mismatches used to create mutations in the RPR and L7Ae, and to alter some codons in POP5. Italic nucleotides refer to the coding sequences for the $(His)_6$ and HA tags, and nucleotides in outlined font refer to amino acids introduced as part of spacers.