Supplementary Methods, Tables and Figures

Characterization of RNA-based and protein-only RNases P from bacteria encoding both enzyme types

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SUPPLEMENTARY METHODS

Reference numbers of genomes, gene loci and growth conditions/metabolic features of bacterial strains employed in this study

Alkalilimnicola ehrlichii MLHE-1:

- NCBI RefSeq: NC_008340.1
- *rnpA* locus: MLG_RS14575
- *rnpB* locus: MLG_RS14985
- HARP-coding gene locus: MLG_RS12925

Growth conditions/metabolic features: facultative chemoautotrophic, haloalkaliphilic bacterium, optimal growth at pH 9.3 (Hoeft et al., 2007).

Halorhodospira halophila SL1:

- NCBI RefSeq: NC_008789.1
- *rnpA* locus: HHAL_RS13455 (the annotated RnpA protein was found to be inactive in the present study; a variant extended by 21 aa at the N terminus, assuming an upstream UUG codon as the authentic start codon, turned out to be active)
- *rnpB* locus: HHAL_RS12815
- HARP-coding gene locus: HHAL_RS11335

Growth conditions/metabolic features: obligately photosynthetic, extremely halophilic and anaerobic purple sulfur bacterium that exhibits autotrophic growth up to saturated NaCl concentrations (Challacombe et al., 2013)**;** laboratory growth at 3 M NaCl (Hoeft McCann et al., 2016).

Thioalkalivibrio nitratireducens DSM 14787:

- NCBI RefSeq: NC_019902.2
- *rnpA* locus: TVNIR_RS18060
- *rnpB* locus: TVNIR_RS18335
- HARP-coding gene locus: TVNIR_RS13675

Growth conditions/metabolic features: obligately chemolithoautotrophic, grows aerobically with reduced sulfur compounds and anaerobically with nitrate as electron acceptor, optimal growth at pH 9.5 to 10.0, moderately halophilic (Sorokin et al., 2003)

Methylacidiphilum infernorum V4:

- NCBI RefSeq: NC_010794.1
- *rnpA* locus: MINF_RS04125 (the annotated, recombinantly expressed RnpA protein was inactive; a variant shortened by 17 aa at the N terminus, utilizing a downstream AUG start codon, turned out to be active in the present study)
- *rnpB* locus: MINF_RS10865
- HARP-coding gene locus: MINF_RS00030

Growth conditions/metabolic features: extremely acidophilic methanotrophic representative of the bacterial phylum Verrucomicrobia; optimal growth at pH 2.0 to 2.5, 60°C in the presence of 25% (v/v) methane as the sole energy source (Hou et al., 2008)

Thermodesulfatator indicus DSM 15286

- NCBI RefSeq: NC_015681.1
- *rnpA* locus: THEIN_RS03190
- *rnpB* locus: THEIN_RS11870
- HARP-coding gene locus: THEIN_RS10515

Growth conditions/metabolic features: thermophilic chemolithoautotrophic sulfate-reducing bacterium; optimal growth at 70°C (Moussard et al., 2004).

Thermodesulfobacterium geofontis OPF15

- NCBI RefSeq: NC_015682.1
- *rnpB* locus: TOPB45_RS08710

Growth conditions/metabolic features: thermophilic chemolithoautotrophic sulfate-reducing bacterium; optimal growth at 83°C (Hamilton-Brehm et al., 2013).

Aquifex aeolicus VF5

- NCBI RefSeq: NC_000918.1
- HARP-coding gene locus: AQ_RS03450 (old locus tag: aq_880)

Growth conditions/metabolic features: thermophilic, chemolithoautotrophic, sulfur- and thiosulfate-reducing bacterium; optimal growth at 85-90°C (reviewed in Wäber and Hartmann, 2019)

Chromosomal DNA preparation

For genomic DNA preparation, ~50 mg cell pellet of *Thermodesulfatator indicus* were resuspended in 570 µL 1x TE buffer supplemented with 0.7 mg/mL lysozyme and incubated for 30 min at 37°C. After addition of proteinase K and SDS to give a final concentration of 100 µg/mL proteinase K in 1% (w/v) SDS, the suspension was incubated for another 15 min at 37°C. Next, NaCl was added to a final concentration of 0.7 M and samples were mixed thoroughly before incubation for 2 min at 65°C. After addition of CTAB/NaCl solution (final concentration: 10% (w/v) CTAB in 0.7 M NaCl), the suspension was mixed thoroughly and incubated for 10 min at 65°C. The chromosomal DNA was extracted successively with chloroform/isoamyl alcohol (24:1), phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) before precipitation with isopropanol. After washing the pellet with 75% (v/v) ethanol the DNA was resuspended in 1x TE buffer.

Total RNA preparation

A 10 OD_{600} aliquot of frozen cell pellet was resuspended on ice in 1.6 mL extraction buffer (10 mM NaOAc, 150 mM sucrose, pH 4.8) and divided equally into two reaction tubes. The following volumes refer to one reaction tube. 75 µL lysozyme (20 mg/mL in 1x TE buffer) was added, followed by incubation for 10 min at room temperature. Next, 40 µL 20% (w/v) SDS were added and mixed by pipetting up and down with subsequent incubation for 2 min at 65°C in a water bath. The following steps were repeated three times: 800 µL acidic phenol (prewarmed to 65°C) were added and the sample was vortexed (1 min) for thorough phase mixing; after incubation for 4 min at 65°C, the sample was shock-frozen in liquid nitrogen, thawed for 2 min at 65°C and vortexed again for 30 s; after centrifugation for 10 min at 15,000 *g*, the upper aqueous phase was transferred to a new reaction tube. After phenol extraction, the aqueous phase was extracted once with an equal volume chloroform and the RNA was precipitated by addition of 0.8 volumes isopropanol, incubation for 1 h at -20°C and centrifugation for 40 min at 15,000 *g* and 4°C. The pelleted RNA was air-dried and redissolved in 1x TE buffer. Total RNA quality was checked by 7% denaturing PAGE and ethidium bromide staining.

Northern blot analysis

Total cellular RNA (7-15 µg per lane) or *in vitro*-transcribed RNA (100-500 fmol per lane) was dissolved in 1x denaturing loading buffer (0.01% [w/v] bromphenol blue; 0.01 [w/v] xylene cyanol; 1.3 M urea; 33% [v/v] formamide; 1x TBE). RNA samples were separated by 8% denaturing (8 M urea) PAGE at 25 mA. After separation the RNA was blotted for 3 h onto a positively charged nylon membrane (Roche Diagnostics) by using a semi-dry blotter at 0.8 mA/cm^2 in transfer buffer (20 mM MOPS, pH 7.0, 5 mM NaOAc, 1 mM EDTA). The

hybridization and detection procedures were carried out according to the DIG system protocols (Roche Diagnostics). For image analysis the ChemiDoc Image System (BioRad) was used.

T7 *in vitro* transcription

Templates for *in vitro* transcription were generated by cloning the RNA expression cassette into a bacterial plasmid vector, either by direct use of PCR fragments as template or by annealing a T7 promoter DNA oligonucleotide to a single-stranded DNA template. The linearized plasmid pSB3'hh (Busch et al., 2000) was used as template for *in vitro* transcription of substrate pre-tRNA^{Gly} from *T. thermophilus*. For *in vitro* transcription of bacterial P RNAs, the *rnpB* genes of *A. ehrlichii*, *H. halophila*, *T. indicus* and *T. nitratireducens* were PCR-amplified from chromosomal DNA and inserted via HindIII/XbaI into vector pSP64 (see Table S4). The T7 promoter sequences were introduced with the forward primers. Plasmids were linearized with XbaI for run-off transcription. The *rnpB* gene of *M. infernorum* embedded into the promoter and terminator sequences of *E. coli rnpB* was commercially synthesized and cloned via EcoRI and HindIII into vector pBluescript II SK(+) (Biomatik Corporation, Canada). For *in vitro* synthesis of *M. infernorum* P RNA, the *rnpB* gene was PCR-amplified using a forward primer that introduced the T7 promoter sequence (see Table S4 for primer sequences). The PCR-fragment was gel-purified and employed as template for *in vitro* transcription with T7 RNA polymerase. For *in vitro* transcription, either 40-80 µg/mL linearized plasmid DNA template, 5 µg/mL PCR template or 80-120 µg/mL DNA oligonucleotides were incubated in 80 mM HEPES pH 7.5, 5-15 mM DTT, 10-40 mM $MgCl_2$, 1 mM spermidine, 0.12 mg/mL BSA, 3.75 mM (each) rNTP, 5% (v/v) DMSO, 1 U/mL pyrophosphatase and 500 to 1,000 U/mL T7 RNA polymerase for 2-6 h at 37°C. Transcription products were separated on 7-10% denaturing PAA gels, RNAs were visualized by UV shadowing and RNA bands were excised from the gel using a scalpel. RNAs were eluted from gel slices by overnight incubation in 1 M NaOAc, pH 5 under shaking at 8°C. After isopropanol precipitation and 75% (v/v) EtOH wash, the RNA pellet was redissolved in an adequate volume of ddH_2O and concentration/purity was analyzed by UV spectroscopy.

Construction of *rnpB* expression plasmids for complementation in *E. coli* BW

For complementation analysis of bacterial *rnpB* genes in *E. coli* BW, the expression vector pACYC_EcPT was constructed that contained the native upstream promoter and downstream terminator sequences of *E. coli rnpB*. For this purpose, vector pACYC177 *E. coli rnpB*-wt (Wegscheid and Hartmann, 2006) was PCR-amplified with primers pACYC_EcPT-for and pACYC_EcPT-rev (introducing SphI and XbaI sites). The amplified DNA was

phosphorylated and circularized by T4 polynucleotide kinase (T4 PNK, NEB) and T4 DNA ligase (NEB), respectively, to yield vector pACYC_EcPT. This plasmid was used to construct the P RNA expression vectors for complementation studies. The *rnpB* genes of *A. ehrlichii*, *H. halophila*, *M. infernorum*, *T. indicus* and *T. nitratireducens* were PCR-amplified from the respective pSP64-based vectors (see "T7 *in vitro* transcription" above) and inserted into pACYC_EcPT via SphI and XbaI. The vectors pACYC_*Minf-Eco* 3'-*rnpB* and pACYC_*Tind-Eco* 3'-*rnpB* were constructed to increase the *in vivo* stability of the encoded heterologous P RNAs (for details, see Fig. 3C of the main text). For their construction, the *rnpB* genes of *M. infernorum* and *T. indicus* were PCR-amplified from vectors pACYC_*Minf-rnpB* and pACYC_*Tind-rnpB*, respectively. The PCR-fragments with the primer-encoded additional nucleotides were inserted via SphI and XbaI in vector pACYC_EcPT. In vectors pACYC_*Tind*-P_{12}-*Tma-rnpB* and pACYC_*Tind*-P_{12}-*Tma-Eco* 3'-*rnpB*, the helix P12 of *T. indicus* P RNA was replaced with the P12 element of *T. maritima* P RNA. This was accomplished by PCR amplification of vector pACYC_*Tind-rnpB* and pACYC_*Tind-Eco* 3'-*rnpB* with phosphorylated primers that introduced the *T. maritima* P12 sequence. The PCR product was circularized using T4 DNA ligase and transformed in *E. coli* DH5α cells. Corresponding primer sequences are listed in Table S5.

Construction of HARP expression plasmids for complementation in *E. coli* BW

For functional analysis of HARP variants in *E. coli* BW (Wegscheid and Hartmann, 2006), the *harp* genes were inserted into shuttle vector pDG148(S/X) (Größringer and Hartmann, 2007), abbreviated as pDG in the following constructs. For construction of pDG:*Aehr-harp*[cHis] the *A. ehrlichii harp* gene was first PCR-amplified from pET-28a(+):*Aehr-harp*[cHis] (abbreviated as pET:*Aehr-harp*[cHis]) and inserted into pJET1.2/blunt (Thermo Fisher Scientific; abbreviated as pJET in Table S5). The *harp* gene was then excised from the resulting vector pJET:*Aehr-harp*[cHis] and inserted non-directionally into pDG148(S/X) using the restriction enzymes SmaI and PaeI. A clone containing the *harp* gene in the desired orientation was selected. For the construction of pDG:*Hhal-harp*[cHis], the *harp* gene of *H. halophila* was amplified from vector pET:*Hhal-harp*[cHis] and the fragment was inserted via SphI and SmaI into vector pDG148(S/X). The expression vector pDG:*Minf-harp*[cHis] for *M. infernorum* HARP was constructed by amplifying the *harp* gene from vector pET:*Minf-harp*[cHis] and inserting the fragment non-directionally into SmaI-linearized pDG148(S/X). A clone containing the *harp* gene in the desired orientation was selected. The vector pDG:*Tind-harp*[cHis] for expression of *T. indicus* HARP was constructed by PCR amplification of the *harp* gene from pET:*Tind-harp*[cHis] and insertion into pDG148(S/X) using the restriction sites for SphI and SmaI. For the construction of pDG:*Tnit-harp* the *harp* gene was PCR-amplified from pET:*Tnit-harp*[cHis] and inserted non-directionally into SmaI-linearized

pDG148(S/X). A clone containing the *harp* gene in the correct orientation with respect to the P_{spac} promoter was used for complementation analysis. Primer sequences are listed in Table S5.

Complementation analysis in *E. coli* BW

For complementation analysis on agar plates, 3 mL LB medium supplemented with 10 mM arabinose and appropriate antibiotics (100 µg/mL ampicillin for pACYC177- and pDG148-based plasmids, 10 µg/mL tetracycline for pBR322-based plasmids) were inoculated with a single colony of *E. coli* BW cells containing expression vectors for bacterial RNase P RNAs or HARPs. Cells were grown to late exponential growth phase at 37°C/200 rpm before 30 µL cell suspension were mixed with 500 µL fresh LB medium, centrifuged for 3 min in a desktop centrifuge at 2,400 *g* at room temperature and washed two times with 500 µL fresh LB medium by repeated resuspension/centrifugation and final resuspension in 500 µL fresh LB medium. Serial dilutions of the washed and resuspended cells were streaked on agar plates containing either 10 mM arabinose or glucose plus appropriate antibiotics (see above). The cell growth was documented after 16, 28 and 42 h incubation at 37°C. For droplet complementation analysis, *E. coli* BW cells containing expression vectors for bacterial RNase P RNA or HARP were grown in 3 mL LB medium supplemented with 10 mM arabinose and appropriate antibiotics (see above) overnight at 37°C/200 rpm. Cells (0.6 OD_{600}) were pelleted for 3 min at 2,400 *g* at room temperature and washed twice with fresh 500 µL LB medium by repeated resuspension and centrifugation. After resuspension in 1 mL LB medium, 10 µL or 20 µL of cells (6 x 10^{-3} or 1.2 x 10^{-2} OD_{600}) were spotted on LB agar plates containing either 10 mM arabinose or glucose plus appropriate antibiotics (see above). Alternatively, a single colony was transferred from an agar plate supplemented with 10 mM arabinose and appropriate antibiotics into 500 µL LB medium. After thorough resuspension, cells were washed twice with fresh 500 µL LB medium by repeated centrifugation and resuspension. 5 x 10 µL of this cell suspension were spotted on LB agar plates containing either 10 mM arabinose or glucose plus appropriate antibiotics. The cell growth was documented after 16 h, 28 h and 42 h incubation at 37°C.

Plasmid construction, expression and purification of recombinant HARPs

Harp genes were inserted into vector pET-28a(+). The *harp* gene from *A. ehrlichii* was PCR-amplified from chromosomal DNA and inserted into pET-28a(+) using XhoI and BspHI for insert cleavage and XhoI and NcoI for vector cleavage (BspHI and NcoI yield identical 5'-overhangs). The resulting plasmid pET-28(+)*Aehr-harp*[cHis] encoded the HARP with a C-terminal His tag for purification. An *A. ehrlichii* HARP variant containing an N-terminal His tag was constructed by PCR amplification of the *harp* gene from pET-28(+)*Aehr-harp*[cHis] and

reinsertion into pET-28(+) between its BlpI (Bpu1102I) and NheI sites. The *harp* gene from *H. halophila* was PCR-amplified from chromosomal DNA and inserted into pET-28a(+) using the restriction enzymes BlpI and NheI, yielding plasmid pET-28a(+)*Hhal-harp*[nHis] encoding an N-terminal His tag. For the synthesis of a *H. halophila* HARP with a C-terminal His tag, the *harp* gene was PCR-amplified from vector pET-28a(+)*Hhal-harp*[nHis] and reinserted into pET-28a(+) using the restriction sites XhoI and NcoI. The *harp* gene from *M. infernorum* was PCR-amplified from a commercially synthesized pUC57-based vector (Biomatik Corporation, Canada) and inserted into pET-28a(+) using the restriction enzymes BspHI/NcoI (see explanation above for *A. ehrlichii* HARP) and XhoI, resulting in plasmid pET-28(+)*Minf-harp*[cHis] encoding the HARP with a C-terminal His tag. A *M. infernorum* HARP variant containing an N-terminal His tag was constructed by PCR amplification of the *harp* gene from pET-28(+)*Minf-harp*[cHis] and insertion into pET-28(+) between the NdeI and BlpI. The *harp* gene from *T. indicus* was PCR-amplified from chromosomal DNA and inserted into pET-28a(+) using the restriction enzymes BlpI and NheI, resulting in plasmid pET-28(+)*Tind-harp*[nHis] encoding the HARP with an N-terminal His tag for purification. A *T. indicus* HARP variant containing a C-terminal His tag was constructed by PCR-amplification of the *harp* gene from pET-28(+)*Tind-harp*[nHis] and insertion into pET-28(+) via NcoI and XhoI. The *harp* gene from *T. nitratireducens* was PCR-amplified from chromosomal DNA and inserted into pET-28a(+) using the restriction enzymes BlpI and NheI. The encoded HARP contained an N-terminal His tag for purification. An *T. nitratireducens* HARP variant containing a C-terminal His tag was constructed by PCR-amplification of the *harp* gene from pET-28(+)*Tnit-harp*[nHis] and insertion into pET-28(+) between the NcoI and XhoI sites. All above described pET vectors have been named based on the ORFs in them (e.g., pET:*Aehr-harp*[cHis], Table S5).

For recombinant HARP expression, all LB media were supplemented with 50 µg/mL kanamycin and 30 or 34 µg/mL chloramphenicol in the case of *E. coli* strains Lemo21(DE3) and Rosetta(DE3), respectively. The HARP-expressing pET-28a(+) derivative plasmids described above were expressed in *E. coli* strain Rosetta(DE3), BL21(DE3) or Lemo21(DE3). 3 mL overnight cultures were inoculated with single colonies from agar plates. The precultures were used to inoculate 500 mL LB autoinduction medium supplemented with with 0.2% (w/v) lactose and 0.05% (w/v) glucose. After incubation for ~20 h at 37°C/180 rpm, cells were harvested by centrifugation (2,500 *g*, 4°C, 30 min) and resuspended in 20 mL NPI-20 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). After cell lysis by sonication, cell debris was pelleted by centrifugation for 45 min at 8,000 *g* and 4°C. The filtrated (0.22 µm filters) supernatant was loaded onto a HisTrap column (HisTrap HP, GE Healthcare) using NPI-20 as loading buffer. For elution of bound proteins, 10-15 column volumes (CV) of a linear gradient from 20-500 mM imidazole in NPI-buffer was applied. Fractions were

analyzed by SDS-PAGE and pure protein fractions were pooled and dialyzed against storage buffer (10 mM Tris-HCl pH 8, 100 mM KCl, 0.1 mM EDTA, 3 mM DTT, 50% [w/v] glycerin, and 10 mM $MgCl_2$) or thrombin cleavage buffer TCB (10 mM Tris-HCl pH 8, 100 mM KCl, 0.1 mM EDTA, 2 mM $CaCl_2$, 10% [w/v] glycerin) overnight at 4°C. Proteins carrying an N-terminal His tag were further processed by Thrombin (GE Healthcare) to remove the tag. Thrombin was added to a final concentration of 1 to 10 U per mg of His-tagged HARP, followed by incubation overnight at 4°C. The suspension was loaded onto a MonoQ column (MonoQ 5/50 GL, GE Healthcare) using TCB as loading buffer. The elution of bound protein was achieved by applying a linear gradient over 15 CV against TCB supplied with 1 M KCl. Protein-containing fractions were dialyzed overnight at 4°C against storage buffer or assay buffer. Removal of the His tag was controlled by Western blot analysis as described in Nickel et al. (2017). If necessary, a gel filtration (HiPrep Sephacryl S-300 HR 26/60, GE Halthcare) step was added according to the protocol provided by the manufacturer.

Analysis of recombinant HARP solubility in *E. coli* BW

E. coli BW strains for recombinant expression of His-tagged HARP enzymes from *A. ehrlichii*, *H. halophila*, *M. infernorum*, *T. indicus* and *T. nitratireducens* were grown overnight in 50 mL cultures in the presence of 10 mM arabinose, 25 µg/mL chloramphenicol and 100 µg/mL ampicillin. Cells were harvested by centrifugation (2,500 *g*, 4°C, 30 min) and cell pellets were resuspended in 1 mL NPI-20 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). Cells were disrupted using the FastPrep System (MP biomedicals) with pre-chilled lysing matrix B for 20 s at a speed of 6.0 (once repeated after 5 min incubation on ice). Cell debris was removed by centrifugation for 3 min at 1,000 *g* and 4°C. The supernatant was then centrifuged for 60 min at 15,000 *g* and 4°C to separate soluble and insoluble protein fractions. The insoluble fraction was resuspended in a volume of TUS buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 8 M urea) corresponding to the volume of the soluble supernatant fraction. The soluble and insoluble protein fractions were incubated separately with 100 µL Protino® Ni-NTA (Machery-Nagel) overnight with rotation at 4°C. After two washing steps with 1 mL NPI-20 buffer (2 min centrifugation at 2,500 *g* and 4°C, followed by resuspension), the NiNTA-bound proteins were eluted with 100 µL NPI-500 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole). Equal volumes of both eluates (soluble and insoluble protein fractions) were boiled (95°C) for 3 min in classical Laemmli buffer, separated on 12% Mini-PROTEAN TGX Stain-Free Protein Gels (BioRad) using the Mini-Protean III device (BioRad) and then transferred to a Trans-Blot Turbo Mini 0.2 µm Nitrocellulose Transfer Packs (BioRad) using the Transblot-Blot SD Semi-Dry Transfer Cell (BioRad) at constant 25 V for 7 min. For immunodetection, the nitrocellulose membrane was (i) incubated with 6x-His Tag monoclonal antibodies (1:15,000 dilution; Thermo Fisher

Scientific) for 60 min at room temperature, (ii) was washed three times (each 10 min) with TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% [w/v] Tween 20, pH 7.6), (iii) was incubated with secondary goat-anti-rabbit-HRP antibodies (1:10,000 dilution; Thermo Fisher Scientific) for 60 min at room temperature, (iv) was again washed three times (each 10 min) with TBST buffer, followed by (v) ECL chemiluminescence detection using a ChemiDoc Image System (BioRad).

Complementation analysis of *rnpA* genes

For complementation studies in *B. subtilis* d7 (Größringer et al., 2006), the bacterial *rnpA* genes were inserted into expression vector pDG148(S/X) (Größringer and Hartmann, 2007). The *rnpA* genes from *A. ehrlichii* and *T. nitratireducens* were PCR-amplified from chromosomal DNA and inserted into pDG148(S/X) using the restriction sites for HindIII and SphI. The *rnpA* genes of *M. infernorum* and *T. indicus* were PCR-amplified from commercially synthesized pUC57-based vectors (Biomatik Corporation, Canada) and inserted into pDG148(S/X) using the restriction enzymes SphI and SmaI. The *H. halophila rnpA* gene was PCR-amplified from chromosomal DNA and inserted into pET-28a(+) via NcoI/XhoI to construct pET-28a(+)*Hhal-rnpA*cHis for RnpA purification. This vector was used to PCR-amplify the *H. halophila rnpA* gene and to insert it between the HindIII and SphI sites of pDG148(S/X). A pBR322-based vector was constructed for expression of *T. indicus* RnpA in *E. coli* BW (Wegscheid et al., 2006). This was accomplished by replacing the *E. coli rnpA* gene in a pBR322-based expression vector (Wegscheid and Hartmann, 2006) with the *T. indicus rnpA* gene employing the Gibson Assembly method.

RnpA expression vectors were introduced into *B. subtilis* strain d7 by electroporation (Xue et al., 1999). After transformation, cells were incubated at 37°C on LB agar plates containing 2% (w/v) xylose, 20 µg/mL kanamycin and 5 µg/mL chloramphenicol. For droplet complementation analysis, a single colony was resuspended in 500 µL LB medium. After two washing steps with fresh 500 µL LB medium involving repeated centrifugation and resuspension, the suspension was dropped (3 x 10 µL per spot, 6 replicates each per plate segment) onto LB agar plates containing 2% (w/v) xylose or 2% (w/v) glucose and appropriate antibiotics (see above). *In vivo* functionality of the RnpA proteins was documented after 12 h, 27 h and 45 h incubation at 37°C. The pBR322-based RnpA overexpression vector was introduced into chemically competent *E. coli* BW cells.

3'- and 5'-RACE

For 3'-RACE analysis, 25 µg of total cellular RNA from *T. indicus* was incubated with 6 U DNase I (NEB) for 50 min at 37°C in a total volume of 50 µL. After acidic phenol-chloroform extraction and ethanol precipitation, the pellet was redissolved in 40 µL ddH_2O and equally

divided into two aliquots. One aliquot was directly used for C-tailing and the other aliquot was treated with T4 PNK (NEB) before C-tailing, taking into account that 2',3'-cyclophosphate ends may be generated during RNA processing. For T4 PNK treatment, the RNA was incubated with 20 U T4 PNK (NEB) in 1x reaction buffer containing 100 µM ATP and 40 U RiboLock RNase inhibitor (Thermo Scientific Fisher) in a total volume of 50 µL for 4.5 h at 37°C, followed by acidic phenol-chloroform extraction, ethanol precipitation and redissolving of the RNA pellet in 20 µL ddH_2O. For C-tailing, both RNA aliquots were combined and incubated for 90 min at 37°C in a 50 µL reaction containing 1x buffer (NEBuffer 2), 10 mM CTP, 40 U RiboLock RNase inhibitor and 4 U poly(U)-polymerase (NEB). After C-tailing, the RNA preparation was extracted with acidic phenol-chloroform, precipitated with ethanol and redissolved in 25 µL ddH_2O. For reverse transcription, 1 µL of a mixture of three different anchor primers (12 pmol each, for sequences see Table S7) was added to 11.5 µL C-tailed total RNA, followed by incubation for 5 min at 65°C before cooling on ice. Next, the solution was adjusted to 1x RT buffer, 625 nM each dNTP, 5 mM DTT and 2 U RiboLock RNase inhibitor (final volume: 19 µL), followed by incubation for 2 min at 42°C to anneal the primers. Then cDNA synthesis was initiated by adding 1 µL SuperScript III enzyme (200 U/µL, Thermo Fisher Scientific) and the sample was incubated for 10 min at 42°C, 30 min at 50°C, 20 min at 55°C and 15 min at 70°C. Subsequently, the RNA was degraded with 2.5 U RNase H (NEB) for 20 min at 37°C. For PCR amplification of *T. indicus* P RNA-based cDNA, a reverse primer specific for the anchor primer sequence and three different forward primers complementary to different regions of the *rnpB* sequence were used (see Table S7 for sequences). After separation on 0.7% agarose gels, prominent PCR products were excised from the gel and extracted using a nucleic acid purification kit (Monarch, NEB). The purified DNA fragments were ligated via TA cloning into a vector (NEB PCR cloning kit) and transformed into 10-ß competent *E. coli* cells (NEB). For each prominent amplification product, several plasmid clones were sequenced.

For 5'-RACE analysis, 50 µg of *T. indicus* total cellular RNA was incubated with 12 U DNase I (NEB) for 50 min at 37°C in a total volume of 100 µL. After acidic phenol-chloroform extraction and ethanol precipitation, the pellet was redissolved in 75 µL ddH_2O and divided into three equal aliquots for adapter RNA ligation by T4 RNA ligase considering that bacterial cellular RNAs may carry 5'-triphosphate, 5'-monophosphate or 5'-hydroxyl ends. Two of these aliquots were pretreated before 5'-adapter ligation as follows: pyrophosphates were removed from 5'-triphosphate ends in a 50 µL reaction with 20 U RppH enzyme (NEB) and 20 U RiboLock RNase inhibitor in 1x buffer (NEBuffer 2) for 60 min at 37°C. 5'-hydroxyl ends were phosphorylated in a 50 µL reaction with 20 U T4 PNK (NEB), 200 µM ATP and 20 U RiboLock RNase inhibitor in 1x buffer (supplied by NEB for T4 PNK) for 120 min at 37°C. The untreated aliquot and the two pretreated samples were pooled and extracted with acidic

phenol-chloroform, precipitated with ethanol and redissolved in ddH_2O. Next, the RNA adapter was ligated to ~4 µg cellular RNA in a 50 µL reaction supplemented with 20 U T4 RNA Ligase 1 (NEB), 1 mM ATP, 15% (w/v) PEG 8000 and 20 U RiboLock RNase inhibitor in 1x buffer (supplied by NEB for T4 RNA Ligase 1) for 180 min at room temperature. After extraction with acidic phenol-chloroform and precipitation with ethanol, the RNA was redissolved in 25 µL ddH_2O. For reverse transcription, 1 µL of a mixture of four different *T. indicus* P RNA-specific primers (each 12 pmol, for sequences see Table S7) were added to 11.5 µL adapter-ligated total RNA, followed by incubation for 5 min at 65°C before cooling on ice. Annealing, cDNA synthesis and RNA degradation were done as described above for 3'-RACE. For PCR amplification of *T. indicus* P RNA-based cDNA, an adapter-specific forward primer and four reverse primers complementary to different regions of the *rnpB* sequence were used (see Table S7 for sequences). Gel purification of the PCR products and subsequent TA cloning was performed as described above for 3'-RACE analysis.

Size exclusion chromatography

Expression and purification of proteins was performed as reported previously (Nickel et al., 2017; Feyh et al., 2021) and detaild above in section "Plasmid construction, expression and purification of recombinant HARPs", using nickel-chelate affinity chromatography (HisTrap HP, Cytiva). The N-terminal His tag was removed from *Tnit*_HARP_nHis and *Hhal*_HARP_nHis by thrombin cleavage (see aforementioned section). Protein concentrations were determined by Bradford assay. Size exclusion chromatography was performed using a Superose 6 10/300 GL (24 mL, GE Healthcare). The column was first equilibrated with 2 column volumes (CV) of crystallization buffer at a maximum flow rate of 0.4 mL/min. Then two column runs with standard proteins (3 proteins per mix, ~0.7 mg each) were performed; mix 1: carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa); mix 2: cytochrome C (12.4 kDa), bovine serum albinum (66 kDa), β-amylase (200 kDa). These elution profiles were used as standard curve to estimate the size/oligomeric state of HARP proteins. For column runs with HARPs, 250 µL of protein lysate were loaded and elution was conducted at a constant flow rate against crystallization buffer (0.4 mL/min). The concentrations of HARPs in the loading samples were as follows: Aq880_cHis, 85 µM; *Minf*_HARP_cHis, 29 µM; *Hhal*_HARP, 677 µM; *Hhal*_HARP_nHis, 44 µM; *Tnit*_HARP, 839 µM; *Tnit*_HARP_nHis, 201 µM; *Tind*_HARP_cHis, 17 µM. The main peak was localized by using the peak integrate function of the UNICORN 5.01 software (GE Healthcare).

Supplementary Tables

Table S1: Activity of bacterial RNase P enzymes

k_{obs} [min^{-1}]	RNA alone	holoenzyme (chimeric)*
E. coli	0.64 ± 0.19	1.77 ± 0.66
A. ehrlichii	1.74 ± 0.34	2.79 ± 1.34
H. halophila	0.97 ± 0.29	1.51 ± 0.8
M. infernorum	0.44 ± 0.08	1.25 ± 0.22
T. indicus	0.09 ± 0.05	0.2 ± 0.12
T. nitratireducens	0.5 ± 0.13	1.04 ± 0.43

*reconstituted with *B. subtilis* RnpA; mean values ± SD.

Table S2: Comparison of folding protocols for P RNAs (n = 2-3)

k_{obs}[min^{-1}]	2' at 85°C (no Mg); 15'at 55°C (+ Mg); 15' at 37°C (+ Mg)	2' at 85°C (no Mg); 30'at 55°C (+ Mg); 15' at 37°C (+ Mg)	- 15' at 55°C (+ Mg); 15' at 37°C (+ Mg)	- 30' at 55°C (+ Mg) 15' at 37°C (+ Mg)
E. coli P RNA	1.62 ± 0.11	2.41 ± 0.16	2.22 ± 0.01	2.57 ± 0.40
T. indicus P RNA	0.23 ± 0.03	0.33 ± 0.03	0.30 ± 0.02	0.36 ± 0.04

Table S3: Temperature-dependent activity of *T. indicus* P RNA

	k_{obs} [min^{-1}]	
	RNA alone	holoenzyme (chimeric)*
E. coli	25°C: 0.59 ± 0.02	25°C: 0.46 ± 0.08
	50°C: 1.02 ± 0.29	50°C: 3.86 ± 3.13
	60°C: 0.14 ± 0.06	60°C: 13.54 ± 8.01
	70°C: 0.002 ± 0.003	70°C: 0.05 ± 0.06
T. indicus	25°C: 0.059 ± 0.004	25°C: 0.08 ± 0.04
	50°C: 0.16 ± 0.03	50°C: 2.18 ± 1.27
	60°C: 0.20 ± 0.22	60°C: 3.13 ± 1.69
	70°C: 0.002 ± 0.002	70°C: 0.46 ± 0.22
T. maritima	25°C: 0.04 ± 0.01	25°C: 0.18 ± 0.09
	50°C: 0.12 ± 0.01	50°C: 3.17 ± 3.08
	60°C: 0.22 ± 0.21	60°C: 2.38 ± 1.48
	70°C: 0.002 ± 0.001	70°C: 0.00 ± 0.00

* reconstituted with *T. maritima* RnpA; mean values ± SD.

Table S4: Primers (in 5' to 3' direction) for the construction of plasmid templates for *in vitro* T7 transcription and sequences of T7 transcripts

	Primer sequences (5' to 3')
A.ehrlichii pSP:*Aehr-rnpB*	AG*A AGC TT*A ATA CGA CTC ACT ATA GGC GCA GGA GTC GGC CGG GCA GCC GC
	AG*T CTA GA*G TGG AGT CGG CCT GTA AGC CGG GTT CTG TCG GGG AC
H. halophila pSP:*Hhal-rnpB*	AG*A AGC TT*A ATA CGA CTC ACT ATA GGA CGG GAT GAG TCG GCC GGA TGG CCG CTG TG
	AG*T CTA GA*G GGT GAG TCG GCC TGT AAG CCG GGT TCT GTC GAG G
T. indicus pSP:*Tind-rnpB*	AG*A AGC TT*A ATA CGA CTC ACT ATA GGA TTT TAA ATG GGG TCG GTC GGA GGT CGC CGG
	AG*T CTA GA*A TAA AAA GGG GCC GGC CAT AAG CCG AGT TCT GTA CC
T.nitratireducens pSP:*Tnit-rnpB*	AG*A AGC TT*A ATA CGA CTC ACT ATA GGC TAC GGG AGT CGG CCG GGC TGT CGC TGC G
	AG*T CTA GA*G TGG GAG CCG GCC TGT AAG CCG GGT TCT GTC GAG G
M. infernorum PCR fragment	AG*T CTA GA*T AAT ACG ACT CAC TAT AGG ACG CAC CGA GGG GTA TGC CGC CGC TCG ACT CC
	CC*G ATA TC*G GCA CCG AGG GGA ATA AGC CGG ATT CTG TGA TCC
pSP stands for pSP64	relevant restriction sites italicized T7 promoter sequence underlined

	T7 transcripts (5' to 3')
A.ehrlichii pSP:*Aehr-rnpB*	GGCGCAGGAGUCGGCCGGGCAGCCGCUGCGGCCCGGGUCGCGAGACCCGGUGCGC GGAGGAAAGUCCGGGCUCCACAGGGCAGGGUGCCAGGUAACGCCUGGGGGGCGUG AGCCCACGGAAAGUGCCACAGAAAACAAACCGCCGAUGGCCCUUCGGGGCACAGG UAAGGGUGAAACGGUGCGGUAAGAGCGCACCGCGCGACUGGUAACAGUUCGCGGC ACGGUAAACCCCACCCGGAGCAAGACCAAAUAGGGGAGCAAUGGCGUGGCCCGCG CUGCUCCCGGGUAGGUUGCUAGAGGCAGGUGGUGACACCUGUCCCAGAGGAAUGG CUGUCCCCGACAGAACCCGGCUUACAGGCCGACUCCACUCUAG
H. halophila pSP:*Hhal-rnpB*	GGACGGGAUGAGUCGGCCGGAUGGCCGCUGUGUCGCAAGGCGACACGGAGGAAAG UCCGGGCUCCACAGGGCAGGGUGCCAGGUAACGCCUGGGGGGCGUGAGCCCACGG AAAGUGCCACAGAAAACAGACCGCCGAUGGCCGCCGCUAGGCGGCACAGGUAAGG GUGAAAAGGUGCGGUAAGAGCGCACCGCGCGACCGGCAACGGUUCGCGGCACGGU AAACCCCACCCGGAGCAAGGCCAAAUAGGGGAGCGAUGGCGUGGCCCGCGCCGCU CCCGGGUAGGCCGCUAGAGGCGUGCGGAGACGCACGUCCCAGAGGAAUGGCCAUC CUCGACAGAACCCGGCUUACAGGCCGACUCACCCUCUAG
T. indicus pSP:*Tind-rnpB*	GGAUUUUAAAUGGGGUCGGUCGGAGGUCGCCGGGGAGUAAUCCUCGGAGGAAAGU CCGAGCUCCACAGGGCAGGGUGCCGGGUAACUCCCGGAGGGGGUAACCCCUGGAA AGUGCCACAGAAAACAGACCGCCCCGUACCCAUUUUCAGGGCGAAUUGAAUCUAU AGACGAUUGAGAAGGUACGUUUAUUUUUAGGGGCUACCCAAUUUGCCUCAAAAAU GGGUGCGGGGUAAGGGUGAAAAGGUGGGGUAAGAGCCCACCGCCUCUCCUGGUAA CAGGAGAGGGCACGGCAAACCCCACCCGGAGAAAGGCCAUGUAGGGGAGCGCUUG AGGGUGCCCCGCCCGAUGCUCCCGGGUAGGCCGCUUGAGCGGUUGGGUAACCAAU CGCCUAGAGGAAUGACCUCCACCCCGAAUGGGGUACAGAACUCGGCUUAUGGCCG GCCCCUUUUUAUUCUAG
T.nitratireducens pSP:*Tnit-rnpB*	GGCUACGGGAGUCGGCCGGGCUGUCGCUGCGCGCAGGCGUGGAGGAAAGUCCGGG CUCCGCAGGGCAGGACGCCAGGUAAUCCCUGGGCGGCGCAAGCCGACGGAAAGUG CCACAGAAAAGAUACCGCCGAUGGGGCCGGUUCCGUCCGGUUCACAGGUAAGGGU GAAAUGGUGCGGCAAGAGCGCACCGCGCGCCUGGCAACAGUGCGUGGCAGGGUAA ACCCCGUCCGGAGCAAGGCCAAAUAGGGGUGCGAUGGUGUGGCCCGCACUGCACC CGGGUAGGCUGCUGGAGGCGCCUGGCGACAGGCGUCGCAGAGGAAUGACAGCCCU CGACAGAACCCGGCUUACAGGCCGGCUCCCACUCUAG

M. infernorum PCR fragment	GGACGCACCGAGGGGUAUGCCGCCGCUCGACUCCCUAUUCUUAUCGAAGU CGAGAGGAAAGUCCGGACUCUUUAAGGCAGCGAUCCACUGGAAACAGUGG GCUUGAAUAGGCGCAAGCCUUUCAAGACGGAGAGUGUCACAGAAAAUAUA CCGCUCAACAAUCUUUGUUGAGUAAGGGUGAAAAGGCGGGGUAAAAGCCC ACCGCAUGAAUGGUAACAUUCAUGGCAGGAAAAACCCAUCGCAGAGCAAG GCCAAGCGGGGACAAGGGUCGCCUUACCCAUCUCAAAUAGCCUUGAGAAG UCCCAGGUAGGCCGCACCGCUUGCCUAAGCGGAUCUCUGAAUAUAAAGAG AUAGACAGAUGGCGGCAGGUCCUAUGUUGGAUCACAGAAUCCGGCUUAUU CCCCUCGGUGCCΔUΔUGAUAUCGG
E. coli pDW98	GGGAUCΔGΔAΔAΔGCUGACCAGACAGUCGCCGCUUCGUCGUCGUCCUCUUCGGGG GAGACGGGCGGAGGGGAGGAAAGUCCGGGCUCCAUAGGGCAGGGUGCCAGGUAAC GCCUGGGGGGGAAACCCACGACCAGUGCAACAGAGAGCAAACCGCCGAUGGCCCG CGCAAGCGGGAUCAGGUAAGGGUGAAAGGGUGCGGUAAGAGCGCACCGCGCGGCU GGUAACAGUCCGUGGCACGGUAAACUCCACCCGGAGCAAGGCCAAAUAGGGGUUC AUAAGGUACGGCCCGUACUGAACCCGGGUAGGCUGCUUGAGCCAGUGAGCGAUUG CUGGCCUAGAUGAAUGACUGUCCACGACAGAACCCGGCUUAUCGGUCAGUUUCAC CUGAUUUAC
	magenta residues: nucleotides added to the annotated P RNA sequence ΔN: nucleotides deleted from the annotated P RNA sequence

Table S5: Primers (in 5' to 3' direction) for the construction of expression vectors

***rnpB* complementation studies**	
pACYC_EcPT	CGC TTC GGC GGG TTT TTG CTT TTT AGA GTC GAC CTG CAG CCC AA GCT TTT GCC ATT C
	GGT TTT TAC *TCT AGA* CCT *GCA TGC* CAG TAT AGA GGG TTT GCG CGC CCT TGT CAC C
A.ehrlichii pACYC_*Aehr-rnpB*	AG*G CAT GC*A GGA GTC GGC CGG G
	AG*T CTA GA*G TGG AGT CGG CCT GTA AGC
H. halophila pACYC_*Hhal-rnpB*	AG*G CAT GC*G GGA TGA GTC GGC CGG
	AG*T CTA GA*G GGT GAG TCG GCC TGT AAG
M. infernorum pACYC_*Minf-rnpB*	AG*G CAT GC*C ACG CAC CGA GGG G
	AG*T CTA GA*A GGC ACC GAG GGG AAT AAG C
T. indicus pACYC_*Tind-rnpB*	AG*G CAT GC*T TTA AAT GGG GTC GGT CGG AGG TC
	AG*T CTA GA*A TAA AAA GGG GCC GGC CAT AAG
T. nitratireducens pACYC_*Tnit-rnpB*	AG*G CAT GC*T ACG GGA GTC GGC CG
	AG*T CTA GA*G TGG GAG CCG GCC TG
M. infernorum pACYC_*Minf-Eco* 3'-*rnpB*	AG*G CAT GC*G AAG CAC CGA GGG GTA TGC CGC CGC
	AG*T CTA GA*A ATC AGG TGA AAC ACC GAG GGG AAT AAG CCG GAT TCT GTG
T. indicus pACYC_*Tind-Eco* 3'-*rnpB*	AG*G CAT GC*G AAG GGG TCG GTC GGA GGT CGC CGG
	AGC T*TC TAG A*AA TCA GGT GAA AGG GCC GGC CAT AAG CCG AGT TCT GTA CC
T. indicus pACYC_*Tind*-P_{12}-*Tma*-*rnpB*	AGG GCA AGG GTG AAA AGG TGG GGT AAG AGC
	CAA CGG GCG GTC TGT TTT CTG TGG CAC TTT CC
T. indicus pACYC_*Tind*-P_{12}-*Tma-Eco* 3'-*rnpB*	AGG GCA AGG GTG AAA AGG TGG GGT AAG AGC
	CAA CGG GCG GTC TGT TTT CTG TGG CAC TTT CC
	relevant restriction sites italicized

***rnpA* expression and complementation studies**	
A. ehrlichii pDG:*Aehr-rnpA*	TTT *AAG CTT* GTG GCG GCC CGA GG
	TTT *GCA TGC* TTA GTG CCT GTT GCC GTC
H. halophila pET:*Hhal-rnpA*[cHis]	TTT *CCA TGG* TTG TCG GCC GAC GAG AGG
	TTT *CTC GAG* GGA GCC ATC GCA TGG CC
H. halophila pDG:*Hhal-rnpA*	TTT *AAG CTT* TTG TCG GCC GAC GAG AGG
	TTT *GCA TGC* TCA GGA GCC ATC GCA TGG
M. infernorum pDG:*Minf-rnpA*	GGA ATT AGC TT*G CAT GC*T CAT ATT TTA AGT TTA TCC CCC
	TTT TTT TTT *CCC GGG* ATG GCT TTT AGA AAT CCG GC
T.indicus pDG:*Tind-rnpA*	TTT *CCC GGG* GTG GCG GCT TAC GGT ATA AAA AAA
	TTT *GCA TGC* TCA ATA ACT TTT ATA ACT ATT TTT CTC ATA CTT TTT
T.nitratireducens pDG:*Tnit-rnpA*	TTT AAG CTT GTG AGC GGG CCA GCT CC
	TTT GCA TGC TCA TTT CAC GTG CTC CAG CAA C
pBR:*Tind-rnpA*	AAA GTT ATT GAC TCT AGA GTC GAC CTG C
	GCC GCC ACG GTC TGT TTC CTG TGT GAA ATT G
	ACA GAC CGT GGC GGC TTA CGG TAT AAA AA
	CGA CTC TAG AGT CAA TAA CTT TTA TAA CTA TTT TTC TCA TAC TTT TTA AAT AAT TCA ACT
pDG stands for pDG148(S/X)and pBR for pBR322	relevant restriction sites italicized

recombinant HARP preparation	
A. ehrlichii pET:*Aehr-harp*[cHis]	TTT *TCA TGA* TGC GCC GAT TCG TGC
	TTT *CTC GAG* ATG GCC GGC ACC CA
A. ehrlichii pET:*Aehr-harp*[nHis]	TTT *GCT AGC* CGC CGA TTC GTG CTG GAC
	TTT *GCT CAG C*CT AAT GGC CGG CAC CCA GG
H. halophila pET:*Hhal-harp*[cHis]	TTT *CCA TGG* ATG CGC CGA TTC GTG C
	TTT *CTC GAG* CCC GGC GGG CTG G
H. halophila pET:*Hhal-harp*[nHis]	TTT *GCT AGC* CGC CGA TTC GTG CTC G
	TTT *GCT CAG C*CT ACC CGG CGG GCT G
M. infernorum pET:*Minf-harp*[cHis]	TTT TTT *TCA TGA* ATG AGA AAG TTT GTT TTA GAC ACC AGC
	TTT T*CT CGA G*AG AAA CTG GCT CTC CGG T
M. infernorum pET:*Minf-harp*[nHis]	TTT TTT T*CA TAT G*AG AAA GTT TGT TTT AGA CAC CAG C
	TTT TTT *GCT CAG C*TT AAG AAA CTG GCT CTC CGG
T. indicus pET:*Tind-harp*[cHis]	TTT *CCA TGG* ATG GCT TTT GAA TTA GAA GTT GAA AG
	TTT *CTC GAG* AGG ACT GTA AAT AAG GCC TTC T
T. indicus pET:*Tind-harp*[nHis]	TTT *GCT AGC* GCT TTT GAA TTA GAA GTT GAA AGA G
	TTT *GCT* CA*G C*TT AAG GAC TGT AAA TAA GGC C
T. nitratireducens pET:*Tnit-harp*[cHis]	TTT *CCA TGG* ATG CGT CGC TTC GTT CTG G
	TTT *CTC GAG* GCC GGA CGT CTT GGG
T. nitratireducens pET:*Tnit-harp*[nHis]	TTT *GCT AGC* CGT CGC TTC GTT CTG GAT ACC A
	TTT *GCT* CA*G CTC AGC* CGG ACG TCT TGG G
pET stands for pET-28(+) or pET-28a(+)	relevant restriction sites italicized

HARP complementation studies	
A. ehrlichii pJET:*Aehr-harp*[cHis]	TTT *CCC GGG* ATG CGC CGA TTC GTG C
	TTT TTT *ATT TAA ATA* TCC GGA TAT AGT TCC TCC TTT CAG
H. halophila pDG:*Hhal-harp*[cHis]	TTT *CCC GGG* ATG CGC CGA TTC GTG C
	AG*G CAT GC*A TCC GGA TAT AGT TCC TCC TTT CAG
M. infernorum pDG:*Minf-harp*[cHis]	TTT TTT TCA TGA ATG AGA AAG TTT GTT TTA GAC ACC AGC
	AGG CAT GCA TCC GGA TAT AGT TCC TCC TTT CAG
T. indicus pDG:*Tind-harp*[cHis]	TTT *CCC GGG* ATG GCT TTT GAA TTA GAA GTT GAA AG
	AG*G CAT GC*A TCC GGA TAT AGT TCC TCC TTT CAG
T. nitratireducens pDG:*Tnit-harp*[cHis]	TTT TCT AGA TGC GTC GCT TCG TTC TGG
	TTT GTC GAC ATC CGG ATA TAG TTC CTC CTT TCA G
pDG stands for pDG148(S/X)	relevant restriction sites italicized

Table S6: DNA oligonucleotides (in 5' to 3' direction) for northern blot analysis

DNA oligonucleotides for the assembly of T7 templates for probe synthesis	
T7 DNA-oligo	TAA TAC GAC TCA CTA TA
A. ehrlichii template	CAG GAG TCG GCC GGG CAG CCG CTG CGG CCC GGG TCG CGA GAC CTA TAG TGA GTC GTA TTA
H. halophila template	GGA TGA GTC GGC CGG ATG GCC GCT GTG TCG CAA GGC GAC ACC TAT AGT GAG TCG TAT TA
M. infernorum template	GGA TCT CTG AAT ATA AAG AGA TAG ACA GAT GGC GGC AGG TCC TAT GTT GGA TCA CAG AAT CCT ATA GTG AGT CGT ATT A
T. nitratireducens template	GGG AGT CGG CCG GGC TGT CGC TGC GCG CAG GCG TGG AGG AAA GTC CTA TAG TGA GTC GTA TTA
underlined: T7 promoter sequence	

PCR primers for the synthesis of T7 templates for probe synthesis	
T. indicus 5´-region reverse	GGG GTC GGT CGG AGG TCG CCG GGG AG
T. indicus 5´-region forward	AGG ATA ATA CGA CTC ACT ATA GGT CTG TTT TCT GTG GCA CTT TCC
E. coli 5S RNA reverse	TGC CTG GCG GCC GTA GCG CGG TGG TC
E. coli 5S RNA forward	TAA TAC GAC TCA CTA TAG GCA GTT CCC TAC TCT CGC ATG GGG AG
underlined: T7 promoter sequence	

Synthetic digoxigenin-labeled DNA probe	
T. indicus 3´-probe	*AGC ATC GGG CGG GGC ACC CTC AAG CGC T*
* digoxigenin attachment positions	

Sequences (in 5' to 3' direction) of northern blot probes	
A. ehrlichii P RNA	GGU CUC GCG ACC CGG GCC GCA GCG GCU GCC CGG CCG ACU CCU G
H. halophila P RNA	GUG UCG CCU UGC GAC ACA GCG GCC AUC CGG CCG ACU CAU CC
M. infernorum P RNA	GGA UUC UGU GAU CCA ACA UAG GAC CUG CCG CCA UCU GUC UAU CUC UUU AUA UUC AGA GAU CC
T. indicus 5´-region	GGU CUG UUU UCU GUG GCA CUU UCC AGG GGU UAC CCC CUC CGG GAG UUA CCC GGC ACC CUG CCC UGU GGA GCU CGG ACU UUC CUC CGA GGA UUA CUC CCC GGC GAC CUC CGA CCG ACC CC
T. indicus 3´-region	AGC ATC GGG CGG GGC ACC CTC AAG CGC T

T. nitrati-reducens P RNA	GGA CUU UCC UCC ACG CCU GCG CGC AGC GAC AGC CCG GCC GAC UCC C
E. coli 5S rRNA	GGC AGU UCC CUA CUC UCG CAU GGG GAG ACC CCA CAC UAC CAU CGG CGC UAC GGC GUU UCA CUU CUG AGU UCG GCA UGG GGU CAG GUG GGA CCA CCG CGC UAC GGC CGC CAG GCA

Table S7: Primers and RNA oligonucleotides (in 5' to 3' direction) for RACE analysis

Primers for 3´-RACE	
anchor primer 1	AGG AGC CAT CGT ATG TCG GGG GGG GGG GGG G**A**
anchor primer 2	AGG AGC CAT CGT ATG TCG GGG GGG GGG GGG G**T**
anchor primer 3	AGG AGC CAT CGT ATG TCG GGG GGG GGG GGG G**C**
anchor-specific reverse primer	AGG AGC CAT CGT ATG TCG
T. indicus rnpB forward primer 1	AGG CAT GCT TTA AAT GGG GTC GGT CGG AGG TC
T. indicus rnpB forward primer 2	GCG GTT GGG TAA CCA ATC
T. indicus rnpB forward primer 3	CAT TTT CAG GGC GAA TTG AAT C

RNA-oligo for 5´-RACE	
RNA adapter	GUC AGC AAU CCC UAA CGA G

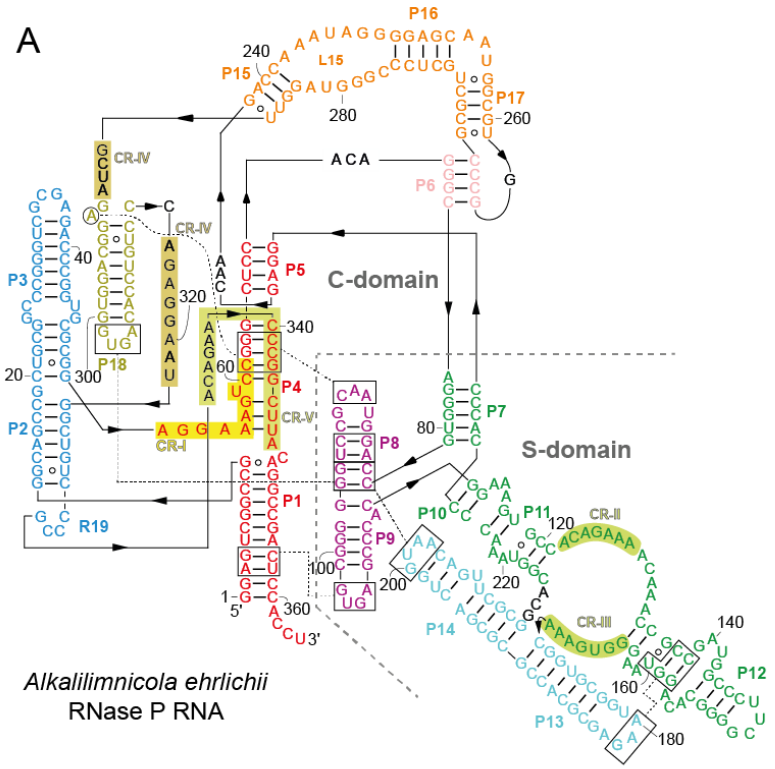
Primers for 5´-RACE	
T. indicus rnpB reverse primer 1	CCT CCG GGA GTT ACC CG
T. indicus rnpB reverse primer 2	CAA ATT GGG TAG CCC CTA AAA ATA AAC G
T. indicus rnpB reverse primer 3	CCT CAA GCG CTC CCC TAC
T. indicus rnpB reverse primer 4	AGT CTA GAA TAA AAA GGG GCC GGC CAT AAG
Adapter-specific forward primer	GTC AGC AAT CCC TAA CGA G

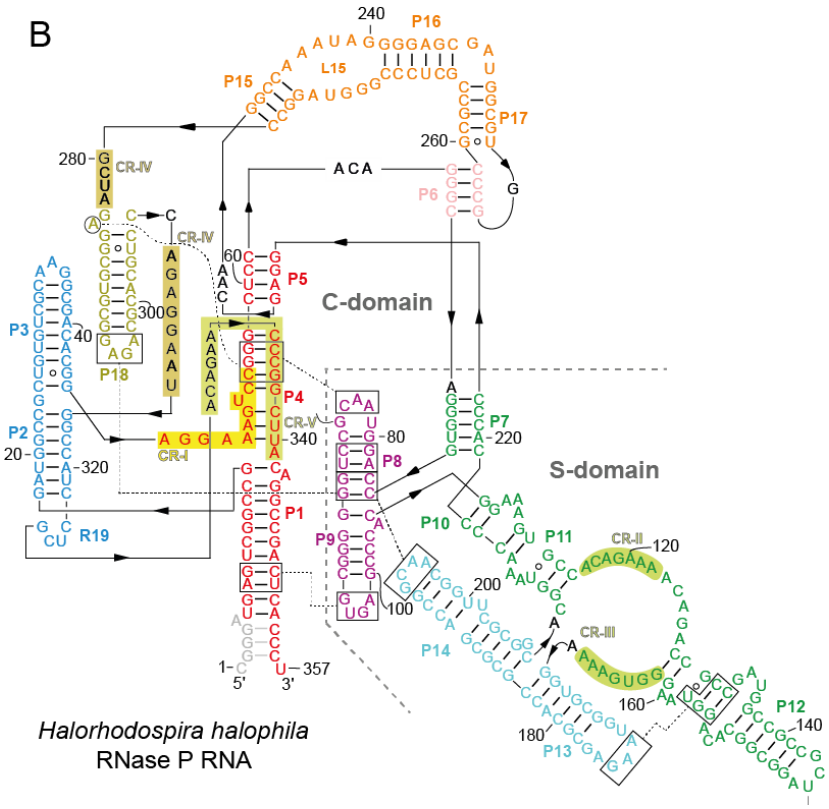
5'-overhangs not complementary to the *Tind rnpB* gene sequence

Table S8: Primers (in 5' to 3' direction) for qRT-PCR analysis

T. indicus rnpA	GAG CGT CTA AGG CGT CGC AG
	ACG TTT CAC CGC ATT TCC TAC C
T. indicus thein2060 (*harp*)	TCT CCC AAT GAG GCT TTC GCC
	ATA CTG CTC TAG CTT TAG GGG G
T. indicus rrf (5S RNA)	CAT ACC GGA GGG GCC ACA CC
	CGC CGA CCT ACT CTC CCA CC

Supplementary Figures





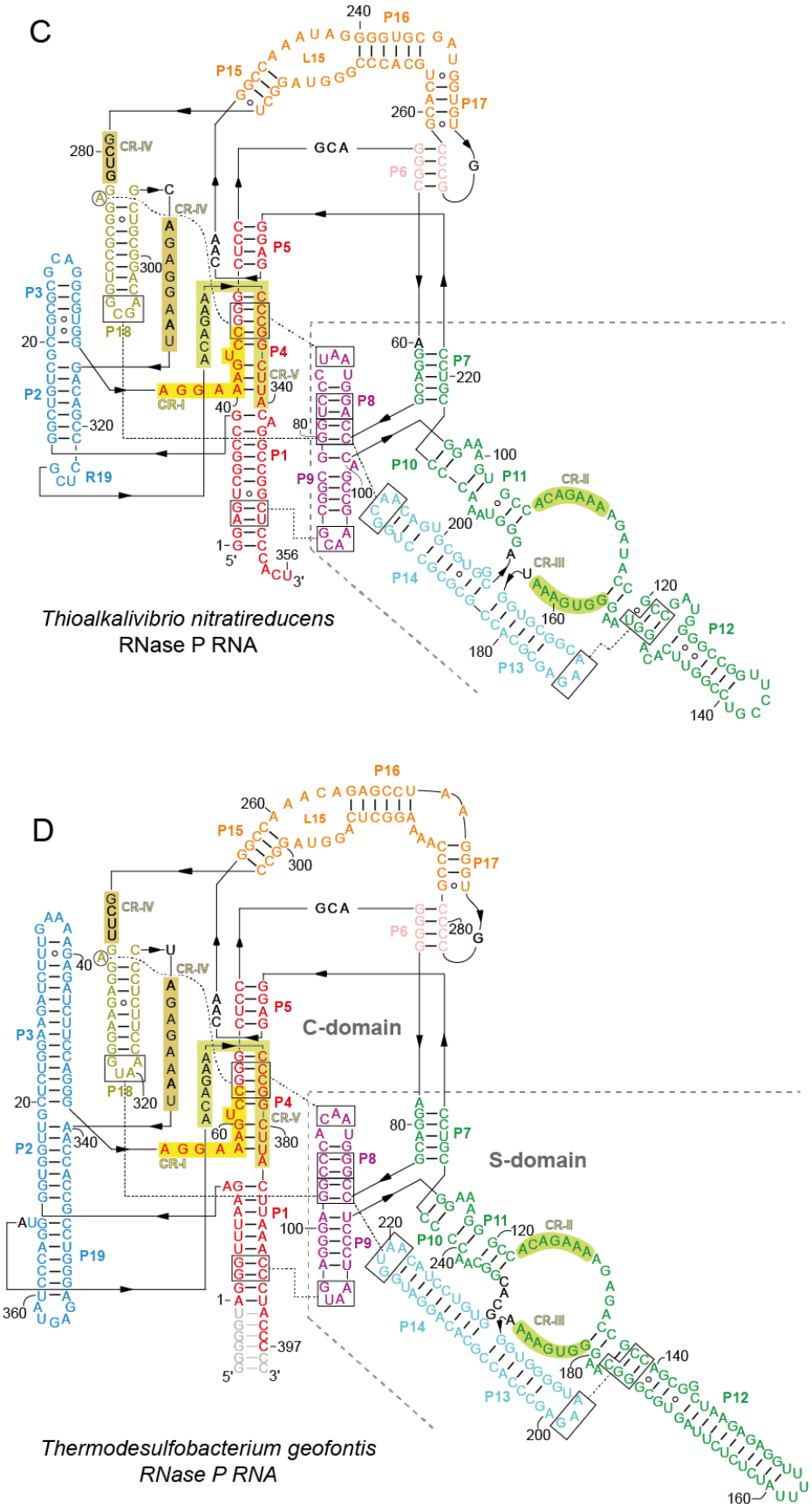


Fig. S1: Predicted secondary structures of RNase P RNAs (P RNAs) from (**A**) *A. ehrlichii*, **(B)** *H. halophila*, (**C**) *T. nitratireducens* and (**D**) *T. geofontis*. The gray nucleotides at the end of helix P1 in

panels B and D, resulting in stabilizing P1 extensions, go beyond the *rnpB* ncRNA sequences annotated in the respective genomes. For details, see legend to Fig. 1 of the main text.

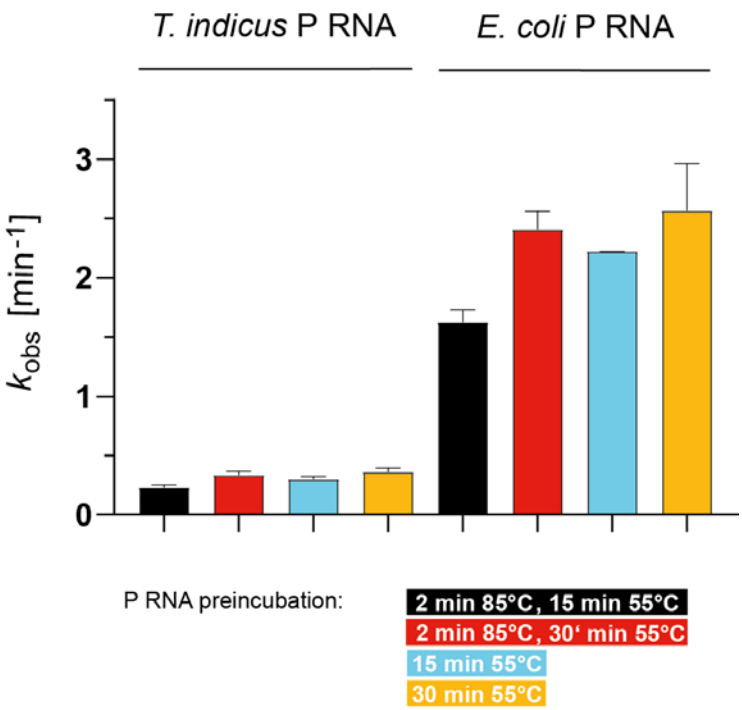


Fig. S2: Different preincubation protocols for *T. indicus* P RNA before processing of 5′-^{32}P-end-labeled pre-tRNA^{Gly} under single turnover conditions at 25°C as in Fig. 2 of the main text. The last preincubation step (15 min at 37°C) was identical in all four preincubation procedures and is therefore not indicated below the graph. Processing assays were conducted in KN buffer supplemented with 100 mM $MgCl_2$. P RNA was either preincubated for (i) 2 min at 85°C in KN buffer, 15 min at 55°C and 15 min at 37°C in KN buffer with 100 $MgCl_2$; or (ii) 2 min at 85°C in KN buffer, 30 min at 55°C and 15 min at 37°C in KN buffer with 100 $MgCl_2$; or (iii) 15 min at 55°C and 15 min at 37°C in KN buffer with 100 $MgCl_2$; or (iv) 30 min at 55°C and 15 min at 37°C in KN buffer with 100 $MgCl_2$. The shown results are based on two independent experiments (± SD).

```
CLUSTAL 2.1 multiple sequence alignment
Gap Open Penalty: 5, Gap Extension Penalty: 0.05


Tind.           MAAYGIKKES------FTKDERLRRRREYERVYRQGKRLSLP-YLRIILAPNQ--LGHSR
Tgeo.           -----MKKEG------LSSKERLRKDEEFQAVFREGKKVWIDAILLIIYKPND--FNYRR
Tth.            -------PAP------GGKLLSLKGDRAFQR-LRKGK-AGRGRYVSVKWLP----AAELR
Aehr.           -----MAARGHGPCA-FPRRRRLTRPQDYRRVFSG-AQKAADRYFTVLARGND--QVGPR
Hhal.           -----MSADER-----FPRGARLLRRREFEGVLRAPEWRTANRYFRVSARQNQ--CGRPR
Tnit.           -----MSGPAPTPGDRFPRSLRLLDGRAFQRVFAR-ARRIPGRRFMLLYRVCAPPLDHPR
Eco.            -----MVKLA------FPRELRLLTPSQFTFVFQQ-PQRAGTPQITILGRLNS--LGHPR
Bsub.           ----------------MKKRNRLKKNEDFQKVFKHGTSVANRQFVLYTLDQPE--NDELR
Minf.           ----MAFRNPAVLNNKFSRKLIAKKKKEIALFFSKGKKFSSSFFILFLLSTEY--QSFPK
                                                           .            :

Tind.           LGLSVSKKVG-NAVKRNYIKRILREVFRRNRDIFPLSHDIVIIPRAKILEIPQ----DKI
Tgeo.           LGIIVSKKIK-KATQRNRVKRLIREFFRRNKDLFPENCDIIIIPHPNLLNFKYRDFVGVV
Tth.            VGIVVSKKVG-KAVVRNKVKRRLREVLRR-----------LHLPQAHLLVVASP---EAR
Aehr.           LGLAISRRVAPRAVDRNRLKRIAREVFRQRQAALG-QRDYVVLARPAARTASKA----RL
Hhal.           LGLAVPKRVLRLAIQRHRVKRIIRESFRVRQGGLP-DHDFVVGVRGAVAEADNA----TL
Tnit.           LGLAISKKHARRAVDRNRIKRVAREAFRARRRSLI-AVDIVVLSRAGIADAGSR----EL
Eco.            IGLTVAKKNVRRAHERNRIKRLTRESFRLRQHELP-AMDFVVVAKKGVADLDNR----AL
Bsub.           VGLSVSKKIG-NAVMRNRIKRLIRQAFLEEKERLK-EKDYIIIARKPASQLTYE----ET
Minf.           VFFAVSKKVN-RACQRNLIKRRMREIFRQHPFFKLSPFAFGWIAKEKALKAPFE----EL
                : : :.::    *  *: :**  *: :                 :

Tind.           KKDLVELFKKYEKNSYKSY---
Tgeo.           KEKLLSLR-KIETSSHDKKIDN
Tth.            EADFAELFRDVVRALRKSGLVQ
Aehr.           HRSLGRLYD-RLN----DGNRH
Hhal.           FSALEELWA-HARGRPCDGS--
Tnit.           RAELDQLLE-HVK---------
Eco.            SEALEKLWRRHCR--LARGS--
Bsub.           KKSLQHLFRKSSLYKKSSSK--
Minf.           KTDMIKLGEKAMQGDKLKI---
                   :  *
```

Fig. S3: Clustal W alignment of RnpA proteins used for complementation analyses in this study. For abbreviations of organism names, see legend to Fig. 4 of the main text. In addition, the RnpA sequences of *Bacillus subtilis* (Bsub) and two other thermophiles, *Thermodesulfobacterium geofontis* (Tgeo) and *Thermus thermophilus* HB8 (Tth) were included in the alignment.

NCBI Reference Sequences: Tnit: WP_015260527.1

```
CLUSTAL O(1.2.4) multiple sequence alignment
Aae       --------MDVFVLDTSVFTNPEIYRTFEEDQRGAMETFIHLALNSR-AEFYMPTSVYTE   51
Aehr      --------MRRFVLDTSVFTNPHCAVQFGEDPLAGVQTFLHLARRCP-AEFYMPLSVYDE   51
Hhal      --------MRRFVLDTSVFTNPDVYLRFDEEPMQAISVFLGLARRAD-AEFYMPGPVYQE   51
Minf      --------MRKFVLDTSIFTNPDVSQQFGDDDSSVFSGFLSLASKAE-ALFYMPLSAYKE   51
Tind      MAFELEVERERLIPDTSVFTNPDVYREFGNSPNEAFANFLELVAKAPNVKVYMPSSVYDE   60
Tnit      --------MRRFVLDTSVFTNPHVFGQFGEDPGDGLRTFLRLAQRCP-AEFFMPLSVYDE   51
                     :: ***:****.    * :.    .  *: *. ..  . .:**  .* *

Aae       MRKIMDVGELWAEFEMVVKIRSPRRFQLTVPADFLYEFIEELRYRINKGLRIAEEHTREA   111
Aehr      FRRMRDLAEMAADFETDVWVRSPRRFSMTIPAEILYEFIHELRGRIDRGLRIAEEHTRQA   111
Hhal      LCNLRSMDLIGAEFETEVYIRSPRRFSMTIPSEVLYEFIEEVRTRIQRGLRIAEEHARQA   111
Minf      LKLIKGTAFFSSDFEFIVHIRSPRKFNLLIPGSVLYDFIDEIRKRIDHGLRIAEEHLKLA   111
Tind      LKKMLKNPKIPPKARAVFRVKSPKKYEIQIPAFLLYELIDDIRQRINKGLRVAEEAVRAS   120
Tnit      FRTMRDLAELAADFETVVWVRSPRRFTLTIPSDILYEFIDEVRTRIDRGLRIAEEHTKRA   111
          :  :     :  . .  . ::**::: : :*. .**::*.::* **::***:***  : :

Aae       SGCEDV----GKLIARLREKYREALRQGILDSKEDVDVLLLAYELDGVLVSADEGLRTWA   167
Aehr      GAATDMR---PELIASLRERYREAMRKGLVDSREDVDAVLLAMELDAELVSADEGMRKLG   168
Hhal      GQAESLP---PELITQLRERYREAMRRGILDSREDIDVVLLAYELDATLVSADEGMRKFA   168
Minf      GPDGGTPIPFDQLVNRLRNRYREALRQGILDSREDVDVILLAYELEAVLVTADEGMKKWA   171
Tind      AHK--SP---DEILNALRRKYREALREGIVDSKEDIELILLALELDGLLLSADRGILNLA   175
Tnit      GAAADMP---PEIITQLRERYREAMRKGLVDSREDVDAVLLALELDAELASADEGMRKLG   168
          .          :::  **.:****:*.*::**:**:: :*** **:. * :**.*: . .

Aae       DKIGIKLIDPKNFKNILESLVRHRF-------------    192
Aehr      NRMGVKLLTADYLRQVMENLGAGH--------------    192
Hhal      ERIGIKLVNPRYLRGVMQNLAGDDPGHAPPCGPDQPAG    206
Minf      DKMGIEILNASYLKDCLKNLIKPEGTGEPVS-------    202
Tind      DKMGIRWIPPEEIRDTIEGLIYSP--------------    199
Tnit      NRMGIKLVTADYLRRVMENLAESQGGGVPKTSG-----    201
          :::*:. :    ::  ::.*
```

Fig. S4: Clustal Omega alignment of HARP proteins analyzed in this study; Aae, *Aquifex aeolicus*. For abbreviations of organism names, see legend to Fig. 5 of the main text. Red letters: residues identical or similar in 5 of the 6 sequences; similarity rules: A = V = I = L; K = R; D = E; F = Y; S = T.

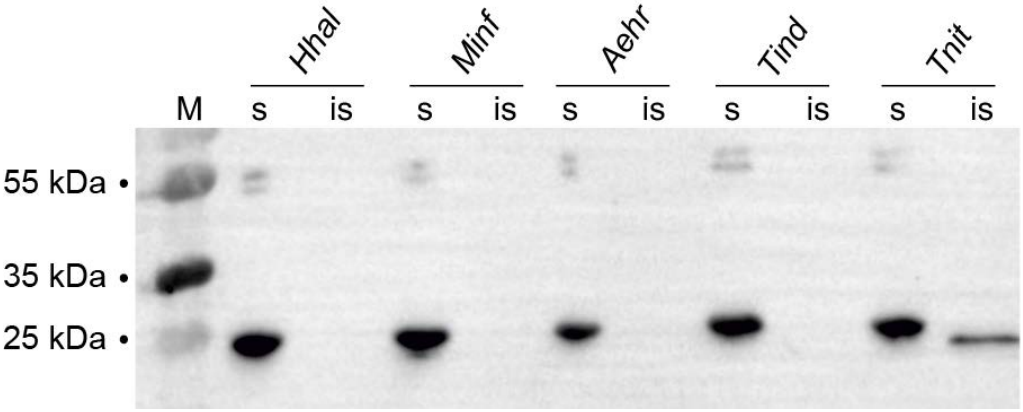


Fig. S5: Solubility of extrachromosomal expressed HARP enzymes from *A. ehrlichii* (*Aehr*), *H. halophila* (*Hhal*), *M. infernorum* (*Minf*), *T. indicus* (*Tind*) and *T. nitratireducens* (*Tnit*) in complementation strain *E. coli* BW. Cells from overnight cultures were lysed before soluble (s) and insoluble (is) protein fractions were separated by centrifugation. After enrichment of the His-tagged HARP proteins by NiNTA batch purification, the levels of recombinant HARP enzymes in both fractions were analyzed by Western blotting with anti-His antibodies. The aberrant migration behavior of the insoluble *Tnit* HARP can be attributed to dissolving the insoluble protein in a denaturing buffer; for details, see Supplementary Methods.

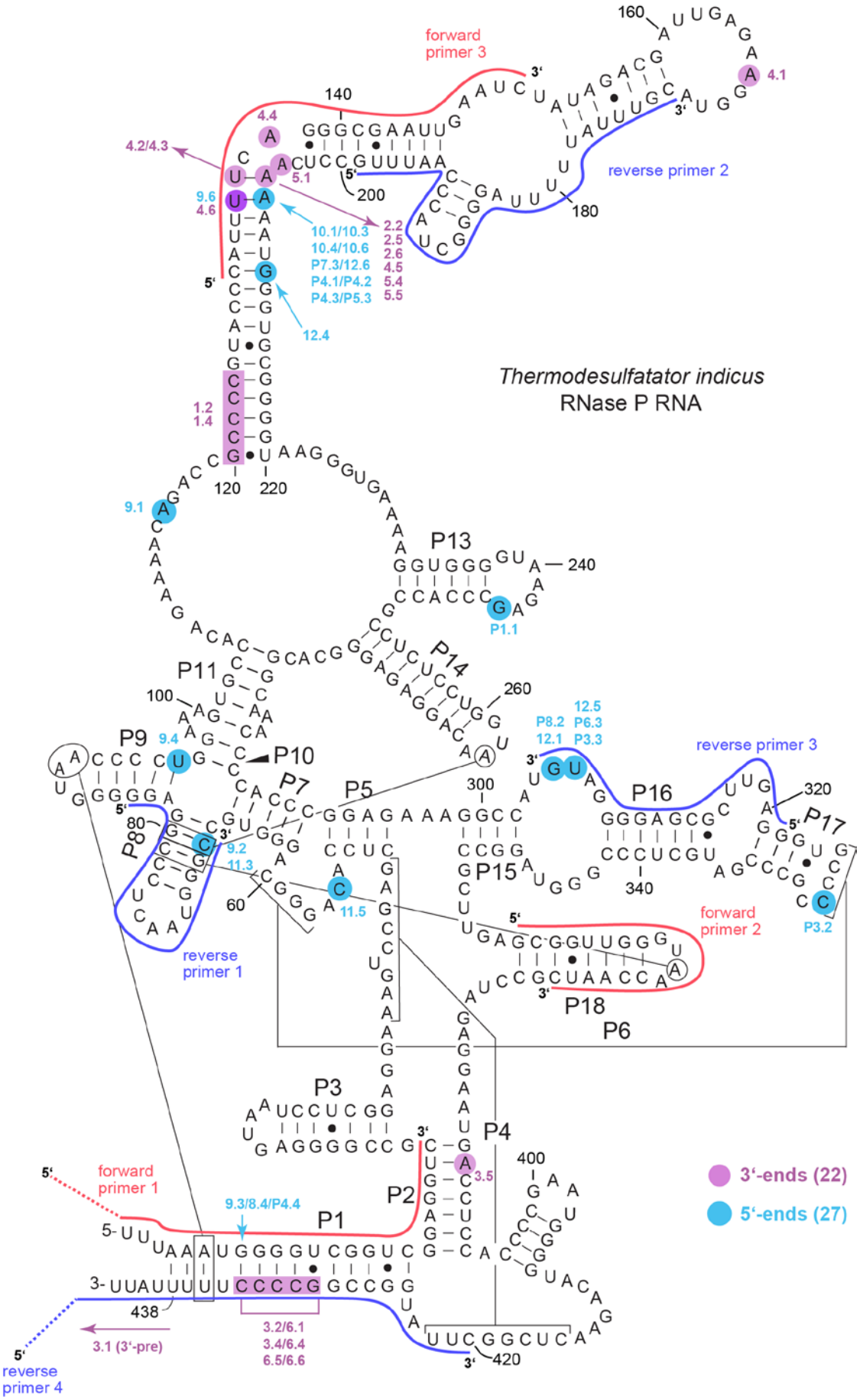
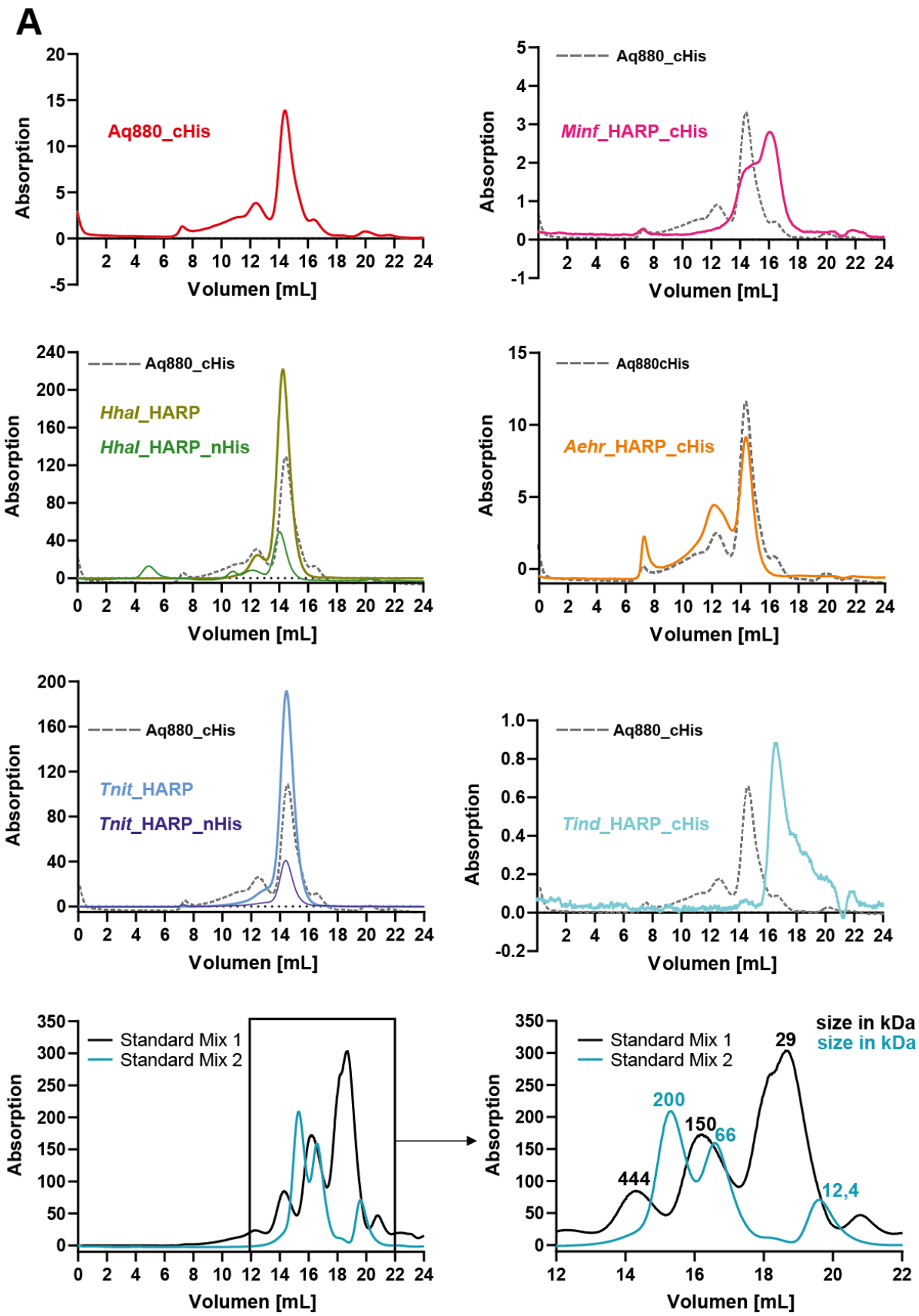

Thermodesulfatator indicus
RNase P RNA
forward primer 3
reverse primer 2
reverse primer 3
forward primer 2
reverse primer 1
forward primer 1
reverse primer 4
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P13
P14
P15
P16
P17
P18
3'-ends (22)
5'-ends (27)

Fig. S6: 3'-ends (22 clones in total) and 5'-ends (27 clones in total) mapped to the *T. indicus rnpB* locus by RACE using total RNA prepared from *T. indicus* cell cultures grown to mid-exponential phase. The annealing sites of the three forward primers and four reverse primers used for 3'- and 5'-RACE, respectively, are shown along the *T. indicus* P RNA sequence; forward primer 1 and reverse primer 4 had eight and seven 5'-terminal nucleotides not complementary to the *T. indicus rnpB* locus (dotted line segments). The mapped nucleotide positions are highlighted in magenta (3'-RACE) and light blue (5'-RACE), along with the clone numbers assigned to these positions. As poly(C) tails were added to RNA 3'-ends before cDNA synthesis, we were unable to differentiate if the original RNA included no or up to 4 of the C residues that follow positions G120 and G430; see also Table 1 of the main text.


A
Absorption
Volumen [mL]
Aq880_cHis
Aq880_cHis
Minf_HARP_cHis
Aq880_cHis
HhaI_HARP
HhaI_HARP_nHis
Aq880cHis
Aehr_HARP_cHis
Aq880_cHis
Tnit_HARP
Tnit_HARP_nHis
Aq880_cHis
Tind_HARP_cHis
Standard Mix 1
Standard Mix 2
size in kDa
29
200
150
66
444
12,4

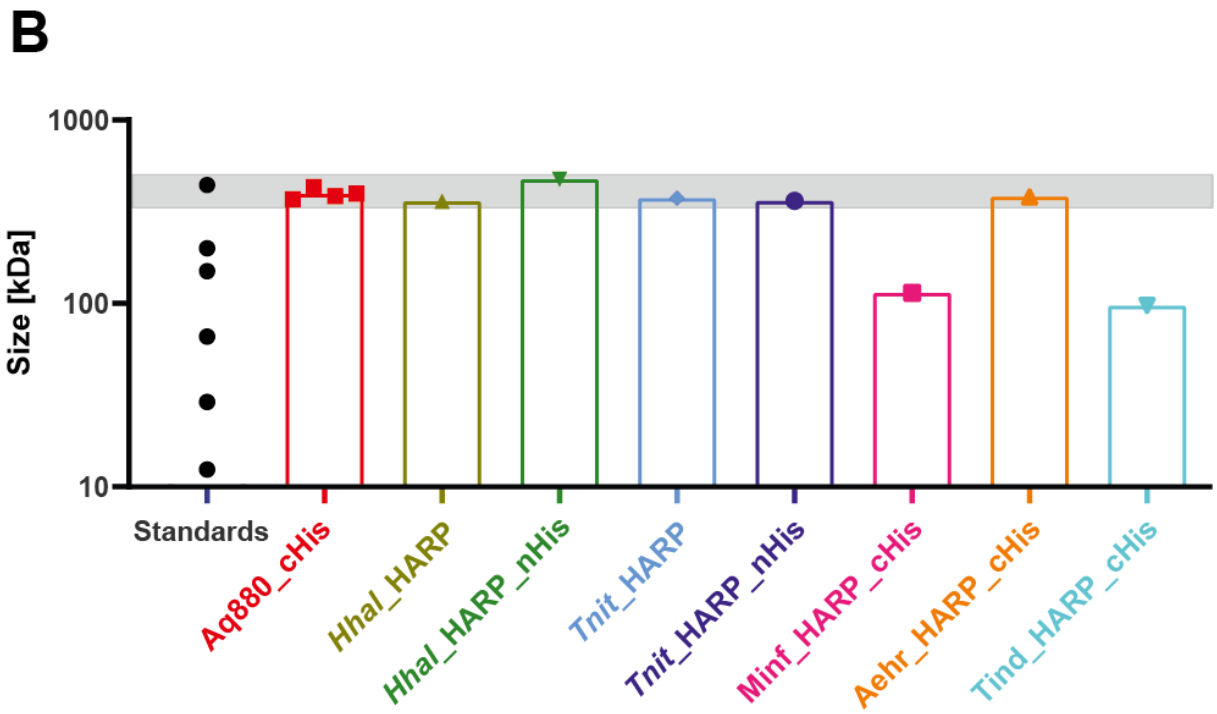


Fig. S7: Analytical size exclusion chromatography (SEC; Superose 6 10/300 GL, column volume 24 mL; GE Healthcare) of recombinant HARP proteins in crystallization buffer (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 10% [w/v] glycerol), using an FPLC ÄKTA basic 10 (GE Healthcare) chromatography system at room temperature. (**A**) Absorption profiles measured at 280 nm; the y-axes are arbitrary relative absorption scales that cannot be directly compared between individual experiments since different protein concentrations were loaded in the shown column runs. The elution volume is shown on the x-axis. Elution profiles of individual HARP proteins are drawn as solid colored lines, with the elution profile of Aq880 shown as stippled gray lines for comparison. *Tnit* and *Hhal* were analyzed with His-tag or after its removal. Elution profiles were similar for the different HARPs, except for *Tind*_HARP_cHis and *Minf*_HARP_cHis for which the main peak was shifted to the right (smaller complexes). The two superimposed profiles at the bottom (entire elution profile on the left, enlarged section on the right) are two calibration runs of the column using the following standard proteins: mix 1: carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa); mix 2: cytochrome C (12.4 kDa), bovine serum albinum (66 kDa), β-amylase (200 kDa). **(B)** Estimated sizes of HARP proteins as inferred from the main peaks in panel A. For Aq880_cHis, four independent runs are plotted including the one illustrated in panel A (upper left graph). It is obvious that *Minf*_HARP_cHis and *Tind*_HARP_cHis eluted as smaller complexes compared to the other HARP variants.

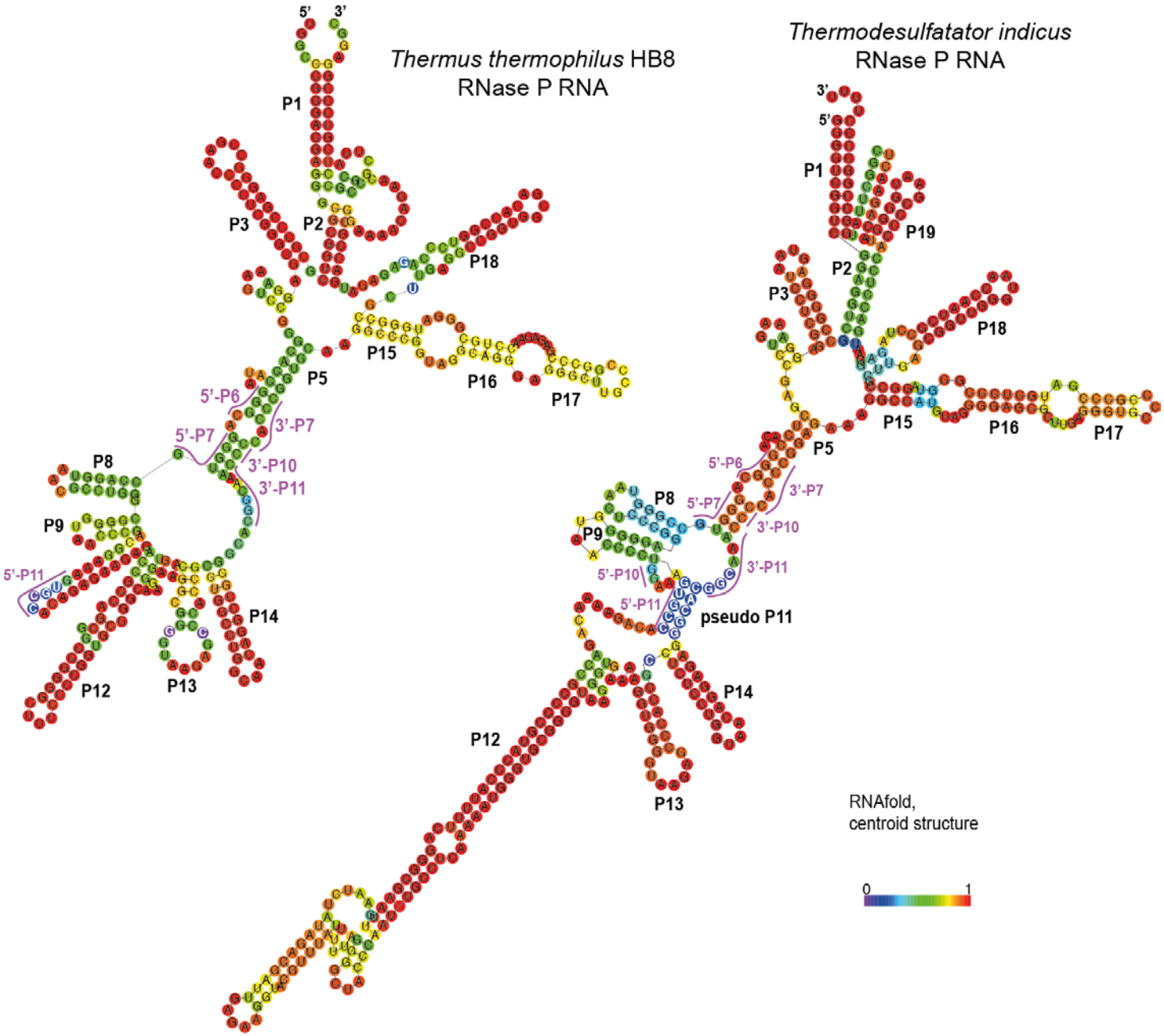


Fig. S8: RNA secondary structure prediction for *T. thermophilus* (adapted from Marszalkowski et al., 2021) and *T. indicus* RNase P RNA using RNAfold with the default settings. The color code of the centroid structure indicates the probability (red: highest; blue: lowest) that a nucleotide is involved in base pairing or is unpaired. The possible folding trap involving pairings between 5'-P6/3'-P7 and 5'-P7/3'-P10/P11 is highlighted by pink lines and letters.

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