tRNA Ligase Catalyzes the GTP-Dependent Ligation of RNA with 3′-Phosphate and 5′-Hydroxyl Termini

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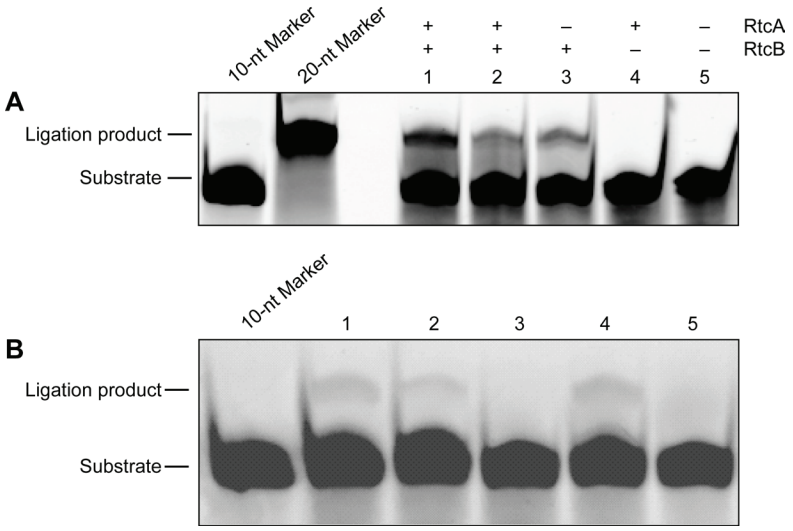

Figure S1. Effect of RNA phosphate cyclase (RtcA), GTP, ATP, and Mg^{2+} on catalysis of RNA ligation by RtcB. (A) Effect of RNA phosphate cyclase (RtcA) and GTP. Lanes 1 and 2 are reaction mixtures (50 µL) consisting of 50 mM Tris–HCl buffer, pH 7.4, containing NaCl (200 mM), $MgCl_2$ (2 mM), DTT (2 mM), ATP (0.3 mM), the 5′ RNA fragment with a 3′-P terminus (200 pmol), and *E. coli* RtcA (1 µM). Reaction mixtures were incubated at 37 °C for 30 min prior to the addition of $MnCl_2$ (2 mM), the 3′ RNA fragment (200 pmol), and *E. coli* RtcB (1 µM). Lanes 1 and 2 are identical reaction mixtures in which the 5′ RNA fragment was incubated with RtcA prior to the addition of RtcB. Lane 3 is a reaction mixture without RtcA, but with GTP (0.3 mM). Lane 4 is a reaction mixture without RtcB. Lane 5 is a reaction mixture without RtcA and RtcB. (B) Effect of GTP, ATP, and Mg^{2+}. Lane 1 is a reaction mixture (50 µL) consisting of 50 mM Tris–HCl buffer, pH 7.4, containing NaCl (200 mM), GTP (0.3 mM), ATP (0.3 mM), $MnCl_2$ (0.5 mM), $MgCl_2$ (1 mM), each RNA fragment (40 pmol), and *E. coli* RtcB (1 µM). Lane 2 is a reaction mixture without $MgCl_2$. Lane 3 is a reaction mixture without GTP. Lane 4 is a reaction mixture without ATP. Lane 5 is a reaction mixture without RtcB.

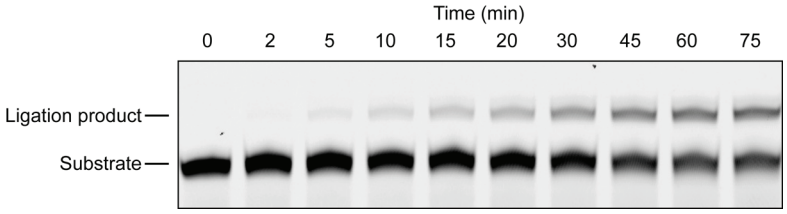

Figure S2. Time-course for the *E. coli* RtcB-catalyzed ligation of the tRNA-mimic substrate. A reaction mixture (200 µL) consisting of 50 mM Tris–HCl buffer, pH 7.4, containing NaCl (200 mM), $MnCl_2$ (0.5 mM), GTP (0.3 mM), annealed oligonucleotides (21 pmol), RNasin® (40 units), and RtcB enzyme (1 µM) was incubated at 37 °C. Aliquots (15 µL) were removed at known times and mixed with an equal volume of RNA gel-loading buffer to quench the reaction.

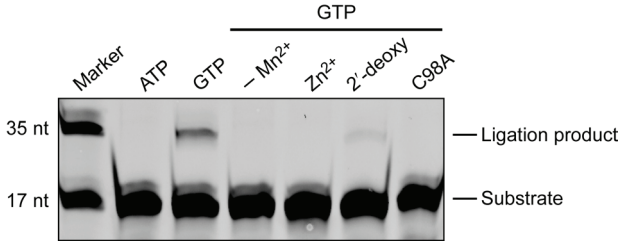

Figure S3. *P. horikoshii* RtcB catalyzed ligation of 3′-P and 5′-OH RNA termini. *P. horikoshii* RtcB requires Mn^{2+} and GTP for ligation. Omission of Mn^{2+} or its replacement with Zn^{2+} did not allow ligation to proceed. The "2′-deoxy" lane is a reaction mixture with the tRNA-mimic substrate that has a 3′-P and 2′-H terminus on its 5′ fragment. The "C98A" lane is a reaction mixture with an RtcB variant in which Cys98 in the predicted metal-binding site was replaced with an alanine residue.

EXPERIMENTAL PROCEDURES

Expression and purification of Escherichia coli RtcB. The *rtcB* gene was amplified from *E. coli* strain MG1655 genomic DNA by PCR using the primers: forward, 5′-TAT GCA TGC ACC ATC ATC ATC ACC ATG GTA ATT ACG AAT TAC TGA CCA C-3′; reverse, 5′-TAT GGA TCC TTA TCC TTT TAC GCA CAC CAC-3′. The PCR product was inserted into the *Sph*I and *Bam*HI sites of a modified pQE-70 vector that encodes LacI for repression of gene expression in the absence of IPTG.[1] After verifying its sequence, the *rtcB*-containing vector was transformed for expression into Keio strain *rtcA*$^{-}$,[2] thereby eliminating any contamination from RNA phosphate cyclase (RtcA) during RtcB purification. Protein was produced by inoculating 0.5 L of Luria–Bertani medium containing ampicillin (100 μg/mL) and growing the culture at 37 °C until OD_{600} = 0.6. IPTG was then added to 0.5 mM, the temperature was reduced to 32 °C, and growth was continued for 2 h. Then, cells were collected by centrifugation. The cell pellet was resuspended in buffer A (50 mM Tris–HCl buffer, pH 7.7, containing 300 mM NaCl, 0.5 mM dithiothreitol (DTT), and 25 mM imidazole), and extracts were prepared by passage through a French pressure cell, followed by centrifugation. The supernatant was loaded onto a column of nickel–nitrilotriacetic acid resin that had been equilibrated with buffer A. The column was washed with 10 column-volumes of buffer A, followed by 10 column-volumes of buffer A containing 40 mM imidazole. The enzyme was eluted with buffer A containing 250 mM imidazole. Purified enzyme was dialyzed against 2 L of 20 mM Tris–HCl buffer, pH 7.4, containing NaCl (200 mM). The concentration of RtcB was determined from the absorbance at 280 nm and a calculated (ExPASy) extinction coefficient of ε_{280} = 52,370 M^{-1} cm^{-1}.

Expression and purification of E. coli RtcA. The *rtcA* gene was amplified from *E. coli* strain MG1655 genomic DNA by PCR using the primers: forward, 5′-TAT GCA TGC TAA AAA GGA TGA TTG CGC TGG-3′; reverse, 5′-TAT GGA TCC TTC AAT GCT CAC CCG CGT TAC-3′. The PCR product was inserted into the *Sph*I and *Bam*HI sites of a modified pQE-70 vector that encodes LacI for repression of gene expression in the absence of IPTG.[1] After verifying its sequence, the *rtcA* clone was transformed into *E. coli* strain BL21 for expression. RtcA was produced, purified, and dialyzed exactly as described for RtcB. The concentration of RtcA was determined from the absorbance at 280 nm and a calculated (ExPASy) extinction coefficient of ε_{280} = 11,460 M^{-1} cm^{-1}.

Expression and purification of P. horikoshii RtcB. A gene encoding the 481-residue RtcB enzyme (Accession #1UC2) from *Pyrococcus horikoshii* was designed with optimized codons for expression in *E. coli* (see sequence below) and synthesized by Integrated DNA Technologies (Coralville, IA). The gene also encoded an N-terminal 6× histidine tag and included a 5′ *Sph*I site and a 3′ *Bgl*II site. *P. horikoshii rtcB* was cloned into the *Sph*I and *Bgl*II sites of a modified pQE-70 vector that encodes LacI for repression of gene expression in the absence of IPTG.[1] After verifying its sequence, *rtcB*-containing vector was transformed into Keio strain *rtcA*$^{-}$,[2] thereby eliminating any contamination from RNA phosphate cyclase (RtcA) during RtcB purification. *P. horikoshii* RtcB was produced and purified exactly as described for *E. coli* RtcB. The stability of the *P. horikoshii* homologue did, however, require a higher salt concentration and was dialyzed against 20 mM Tris–HCl buffer, pH 7.4, containing NaCl (300 mM). Protein concentrations were determined from absorbance readings at 280 nm using a calculated (ExPASy) extinction coefficient of ε_{280} = 62,340 M^{-1} cm^{-1}.

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5'-GCATGCTGAG CTCTCATCAC CATCACCATC ACAGCTCTGG TGAAAATCTG TATTTTCAGA
   TGGTTGTGCC ACTGAAACGT ATCGATAAAA TTCGCTGGGA AATCCCGAAA TTCGACAAGC
   GTATGCGCGT GCCTGGGCGT GTTTACGCGG ATGAAGTATT ATTGGAAAAA ATGAAAAATG
   ACCGCACCTT GGAGCAGGCG ACCAACGTGG CGATGTTGCC AGGAATCTAC AAATATTCGA
   TTGTGATGCC AGACGGCCAC CAGGGCTACG GCTTCCCCAT TGGCGGAGTG GCGGCCTTCG
   ATGTTAAAGA AGGTGTTATC AGTCCGGGCG GTATCGGATA TGACATCAAT TGTGGTGTGC
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   ATACGCTGTT TAAAAATGTT CCATCCGGTG TAGGCTCTCA GGGCCGTATT AAACTGCATT
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   CCACCTGTCA CGGGGCTGGG CGTGTTCTGT CTCGTAAAGC GGCAACGCGT CAATATCGCG
   GTGACCGCAT TCGTCAGGAG CTCCTTAATC GCGGTATTTA CGTGCGTGCC GCTTCTATGC
   GTGTTGTGGC GGAGGAAGCT CCAGGGGCGT ATAAAAACGT TGACAATGTG GTTAAAGTGG
   TTAGCGAAGC AGGCATTGCC AAACTCGTTG CACGTATGCG CCCGATCGGT GTAGCGAAAG
   GCTAAAGATC T-3'
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Ligation assay using single-stranded RNA as substrate. Ligation assays with single-stranded RNA as the substrate used two 10-base oligonucleotides that were synthesized by Integrated DNA Technologies. The 5′ RNA fragment had a 6-carboxyfluorescein (FAM) label on its 5′ end and was phosphorylated on its 3′ end. The 3′ RNA fragment had hydroxyl groups on each end. The sequence of the 5′ fragment was FAM–5′-AAAUAACAAA-3′–P and the sequence of the 3′ RNA fragment was 5′-AAAUAACAAA-3′.

Ligation reactions with RNA phosphate cyclase (RtcA) were performed in 50-μL solutions consisting of 50 mM Tris–HCl buffer, pH 7.4, containing NaCl (200 mM), $MgCl_2$ (2 mM), DTT (2 mM), ATP (0.3 mM), the 5′ RNA fragment containing a 3′-P (200 pmol), and *E. coli* RtcA (1 μM). Reaction mixtures were incubated at 37 °C for 30 min prior to the addition of $MnCl_2$ (2 mM), the 3′ RNA fragment (200 pmol), and *E. coli* RtcB (1 μM). Reactions were incubated for 1 h at 37 °C prior to the addition of an equal volume of RNA-loading buffer. Reaction mixtures were boiled for 2 min before an aliquot (12 μL) was loaded onto an 18% w/v urea–polyacrylamide gel (SequaGel, National Diagnostics, Atlanta, GA). Loaded gels were subjected to electrophoresis at 230 V (constant) for 40 min, and then scanned with a Typhoon FLA 9000 imager (GE Healthcare) using the FAM setting.

After RtcA was found to be unnecessary for ligation, the 50-μL reaction mixtures consisted of only 50 mM Tris–HCl buffer, pH 7.4, NaCl (200 mM), $MnCl_2$ (0.5 mM), GTP (0.3 mM), each RNA fragment (40 pmol), and *E. coli* RtcB (1 μM).

Annealing RNA oligonucleotides to create a broken tRNA mimic. A broken tRNA mimic was created by annealing two single-stranded RNA oligonucleotides synthesized by Integrated DNA Technologies. For routine ligation assays, the sequence of the 5′ fragment was FAM–5′-ACUCCGAUAUCACGCUU-3′–P, and the sequence of the 3′ fragment was

5′-UCACCGUGAUAUCGGAGU-3′. Oligonucleotides with different end groups were used only in assays that probed the specificity of RtcB. Annealing was performed in 50 μL of 30 mM HEPES buffer, pH 7.5, containing $MgCl_2$ (2 mM), KCl (100 mM), and RNasin® from Promega (Madison, WI) (20 units), and each oligonucleotide (0.35 nmol). Annealing solutions were incubated at 95 °C for 2 min, and then at 40 °C for 2 h.

Ligation assay using a tRNA mimic as substrate. The tRNA substrate was created by annealing two single-stranded RNA oligonucleotides that were synthesized by Integrated DNA Technologies. For routine ligation assays, the sequence of the 5′ fragment was FAM–5′-ACUCCGAUAUCACGCUU-3′–P, and the sequence of the 3′ fragment was 5′-UCACCGUGAUAUCGGAGU-3′. The 3′ RNA fragment had hydroxyl groups at each end. Oligonucleotides that had different end structures were used only for assays that probed the specificity of RtcB for RNA termini. The two RNA oligonucleotides were annealed in a 50-μL solution that consisted of 30 mM HEPES buffer, pH 7.5, containing $MgCl_2$ (2 mM), KCl (100 mM), each RNA oligonucleotide (0.35 nmol), and recombinant ribonuclease inhibitor (RNasin®, Promega, Madison, WI; 20 units). This mixture was incubated at 95 °C for 2 min, and then at 40 °C for 2 h.

Ligations with *E. coli* RtcB were performed in 50-μL solutions consisting of 50 mM Tris–HCl buffer, pH 7.4, containing NaCl (200 mM), $MnCl_2$ (0.5 mM), GTP (0.3 mM), annealed oligonucleotides (21 pmol), RNasin® (20 units), and RtcB enzyme (1 μM). Reaction mixtures were incubated at 37 °C for 1 h prior to the addition of an equal amount of RNA gel-loading buffer. The reaction mixtures were boiled for 2 min before 12 μL was loaded onto an 18% w/v urea–polyacrylamide gel (SequaGel). Gels were subjected to electrophoresis at 230 V (constant) for 40 min, and then scanned with a Typhoon FLA 9000 imager at the FAM setting. Studies with nonhydrolyzable GTP analogues were performed by using a nonhydrolyzable GTP test kit (Jena Bioscience, Jena, Germany). Studies of GTP titration reactions used half of the quantity of annealed oligonucleotides, and reactions were incubated at 37 °C for 20 min before 24 μL, containing an equal amount of RNA-gel loading buffer, was loaded on an 18% w/v urea–polyacrylamide gel (SequaGel). Exact GTP concentrations were calculated from absorbance readings at 252 nm using an extinction coefficient of 13,700 M^{-1} cm^{-1}.

Ligations with *P. horikoshii* RtcB were performed in 50-μL solutions that consisted of 50 mM Tris–HCl buffer, pH 7.4, containing NaCl (500 mM), $MnCl_2$ (0.5 mM), GTP (50 μM), annealed oligonucleotides (10.5 pmol), RNasin® (20 units), and *P. horikoshii* RtcB (1 μM). Assay mixtures were incubated at 55 °C for 3 h before the addition of an equal volume of RNA-loading buffer. (*P. horikoshii* grows optimally near 100 °C. Accordingly, the *P. horikoshii* ligase is likely more active at temperatures >55 °C. Higher temperatures are, however, above the melting temperature of our annealed oligonucleotides.) Ligation reactions with *P. horikoshii* RtcB were subjected to electrophoretic analysis as described for reactions with *E. coli* RtcB.

■ REFERENCES

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(2) Baba, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., and Wanner, B. L. (2006) *Mol. Syst. Biol. 2*, 2006.0008.