YvcI from *Bacillus subtilis* has *in vitro* RNA pyrophosphohydrolase activity

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Supporting Information

Contains Supporting Methods, Supporting Figures 1-6 and Supporting Tables 1-4.

SUPPORTING METHODS

Plasmid construction

The genes of YvcI (*yvcI*), BsRppH (*rppH*), YjhB (*yjhB*), MutT (*mutT*), NudF (*nudF*) and YmaB (*ymaB*) were amplified from genomic DNA from *B. subtilis* 1A1 isolated using a published method (1). *Xba*I and *Xho*I restriction sites were introduced during PCR amplification using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) with the primers Fwd-YvcI/Rev-YvcI, Fwd-BsRppH/Rev-BsRppH, Fwd-NudF/Rev-NudF and Fwd-YmaB/Rev-YmaB (Table S2). The amplified PCR products were treated with *Xba*I and *Xho*I (both Thermo Scientific) and cloned into pET-28a(+) (Novagen), resulting in the plasmids pET-28a-YvcI, pET-28a-BsRppH, pET-28a-NudF and pET-28a-YmaB. Due to solubility issues, YjhB and MutT were expressed as maltose binding protein fusions. *yjhB* and *mutT* amplified with the primers Fwd-YjhB/Rev-YjhB and Fwd-MutT/Rev-MutT were cloned into 2M-T (Addgene plasmid #29708; 2M-T was a gift from Scott Gradia) by ligation independent cloning (LIC). In brief, 1 µg 2M-T in 1× *Ssp*I buffer was treated with 10 U *Ssp*I at 37°C for 30 min. The linearized vector was gel purified and used for the LIC reaction: in separate reactions, 150 ng of the vector, or the insert(s), respectively, in 1× T4 DNA polymerase buffer plus 5 mM DTT and 2.5 mM dCTP (insert) or 2.5 mM dGTP (vector) were treated with 3 U T4 DNA polymerase at 22°C for 30 min. After heat inactivation at 75°C for 20 min, 2 µl (each) of the LICed insert and LICed vector were combined in 10 µl and incubated at 20°C for 10 min resulting in the plasmids 2M-T-YjhB and 2M-T-MutT. All mutants of YvcI were generated by site-directed mutagenesis as described in (2). The primer for the introduction of the respective mutations are listed in Table S2. The resulting linearized plasmids pET-28a-YvcI-E49A, -E49Q, -E52Q, -E53Q, -E49Q-E52Q, -E49Q-E52Q, -E52Q-E53Q, were ligated (T4 DNA ligase, Thermo Scientific). *E. coli* DH5α was used for subcloning. Following Sanger sequencing, all plasmids (Table S4) were transformed into *E. coli* BL21 Star (DE3) (Thermo Scientific).

Protein expression and purification

The expression and affinity purification of YvcI (mutants), BsRppH and YmaB from *B. subtilis* and NudC from *E. coli* were performed as described (3,4). HisTrap buffer A (50 mM Tris-HCl pH7.5, 0.3 M NaCl, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 5% glycerol, 5 mM imidazole, 1 tablet per 1 l complete EDTA-free protease inhibitor cocktail (Merck)) and HisTrap buffer B (HisTrap buffer A with 500 mM imidazole) were used. For the expression of YjhB, MutT and NudF from *B. subtilis*, *E. coli* BL21 Star (DE3) harboring the respective plasmid were induced between OD_{600} 0.6-08 with 0.1 mM IPTG. The cells were chilled for 30 min at 4°C and incubated at 17°C for 17 h. The purification by affinity chromatography using HisTrap columns (GE Healthcare) was performed as described (3). Fractions containing the protein of interest were concentrated in a centrifugal filter (10 kDa MWCO,

Amicon, Merck) and the buffer exchanged to Buffer G (50 mM Tris-HCl pH7.5, 300 mM NaCl, 0.1 DTT). For further purification, size-exclusion chromatography was performed using a Superdex 200 10/300 GL column (GE Healthcare) in Buffer G. The purity of all proteins was analyzed by SDS-PAGE (Coomassie staining) and the concentration determined by a BCA protein assay (Thermo Scientific). All proteins were stored in 50% glycerol at –20°C.

Gel electrophoresis

Classical denaturing polyacrylamide gel electrophoresis (PAGE) (5) was used to separate RNA.

SUPPORTING FIGURES

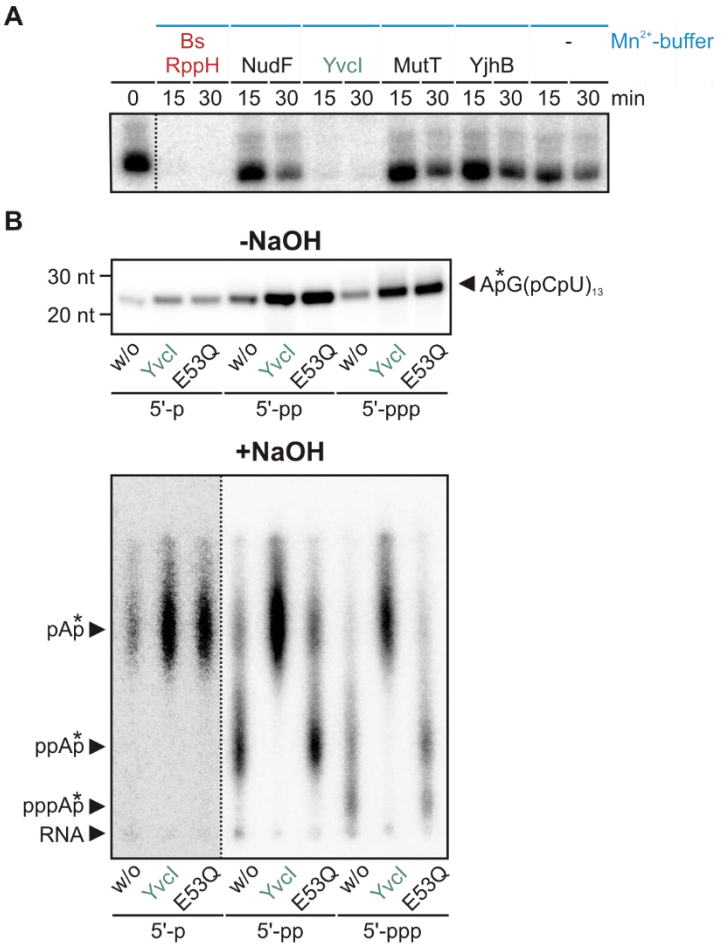

Figure S1. RNA pyrophosphohydrolase activity of YvcI. (**A**) YvcI and BsRppH efficiently remove phosphate from ppp-RNA in presence of Mn^{2+}. ppp-*gapB* sRNA-like 5'-terminal fragment (75mer, 5'-labeled, 0.5 µM) was incubated with Nudix hydrolases (0.5 µM each) in 1× Mn-buffer (2 mM Mn^{2+}). The dotted line indicates where different parts of the gel were combined to facilitate interpretation. 18% seqPAGE, phosphor imaging of radioactivity. (**B**) Conversion of 5'-ppp- and 5'-pp-RNA to 5'p-RNA by YvcI. p/p/p-RNA (p-, pp-, ppp-AG$(CU)_{13}$, with the ^{32}P as part of the phosphate (*) between the first and the second nucleotide) was incubated in absence (w/o) or presence of YvcI, or YvcI-E53Q, respectively (2 µM each) in 1× Mn-buffer (2 mM Mn^{2+}). The reaction products were subjected to alkaline hydrolysis (lower part) or not (upper part). Analysis for samples hydrolyzed with sodium hydroxide by PEI-cellulose TLC and for non-hydrolyzed samples by 15% PAGE, phosphor imaging of radioactivity.

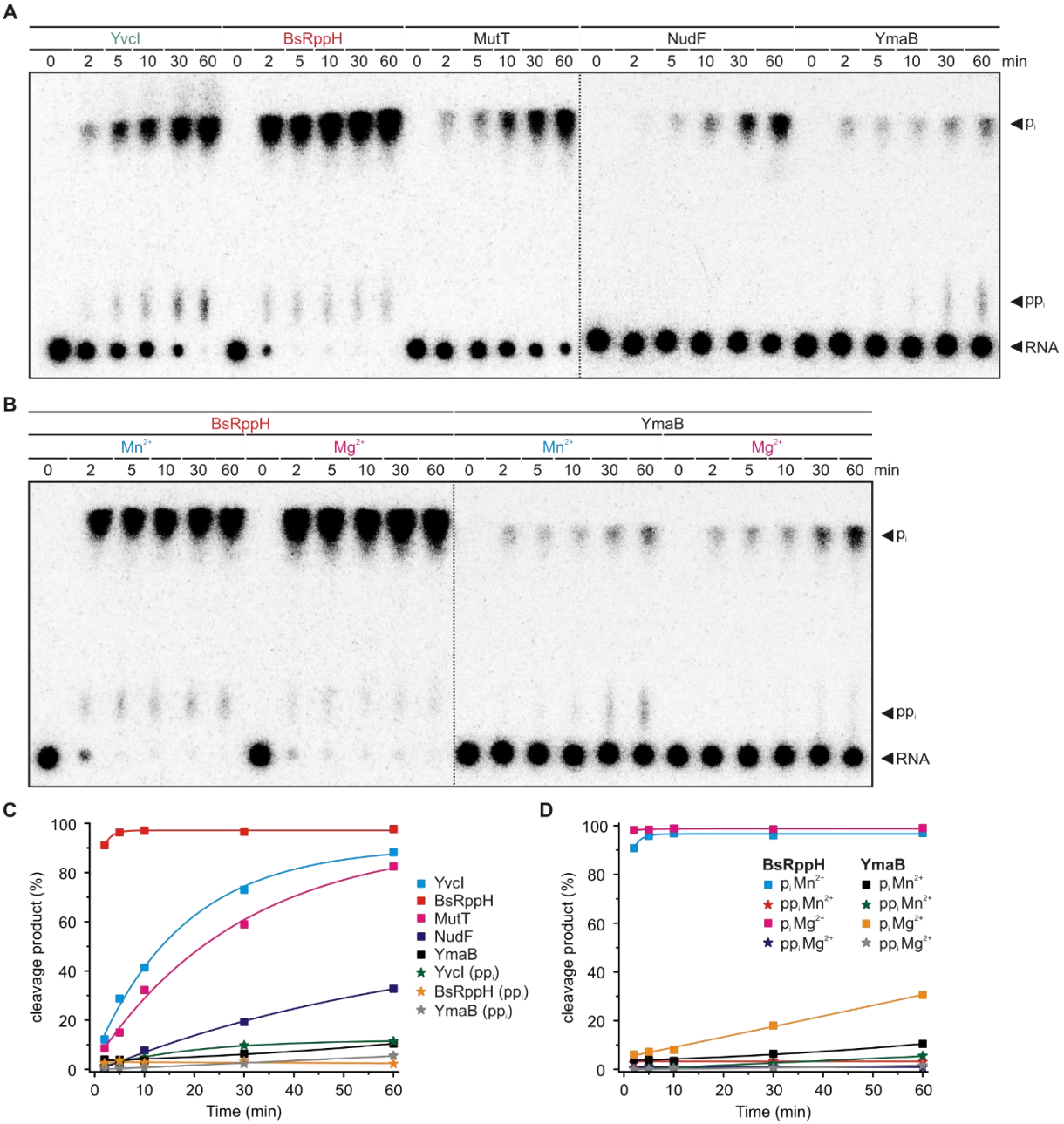

Figure S2. Comparison of the RNA pyrophosphohydrolase activity of *B. subtilis* Nudix hydrolases. (**A**) More Nudix hydrolases than BsRppH (6) show RNA pyrophosphohydrolase activity using manganese (Mn^{2+}) as cofactor. ppp-RNA (AGU 5S rRNA, 5'-labeled, 0.5 µM) was incubated with YvcI, BsRppH, MutT, NudF, or YmaB (2 µM each, 0.1 µM for BsRppH) in 1× Mn-buffer (2 mM Mn^{2+}). PEI-cellulose TLC, phosphor imaging of radioactivity. (**B**) Comparison of the RNA pyrophosphohydrolase activity of BsRppH and YmaB. Procedure as for (**A**). 1× Mg-buffer or 1× Mn-buffer (2 mM Mg^{2+} or Mn^{2+}) were used as reaction buffer. (**C**) Quantification of the cleavage products from (**A**). (**D**) Quantification of the cleavage products from (**B**). The dotted lines indicate where different parts of the different plates were combined to facilitate interpretation.

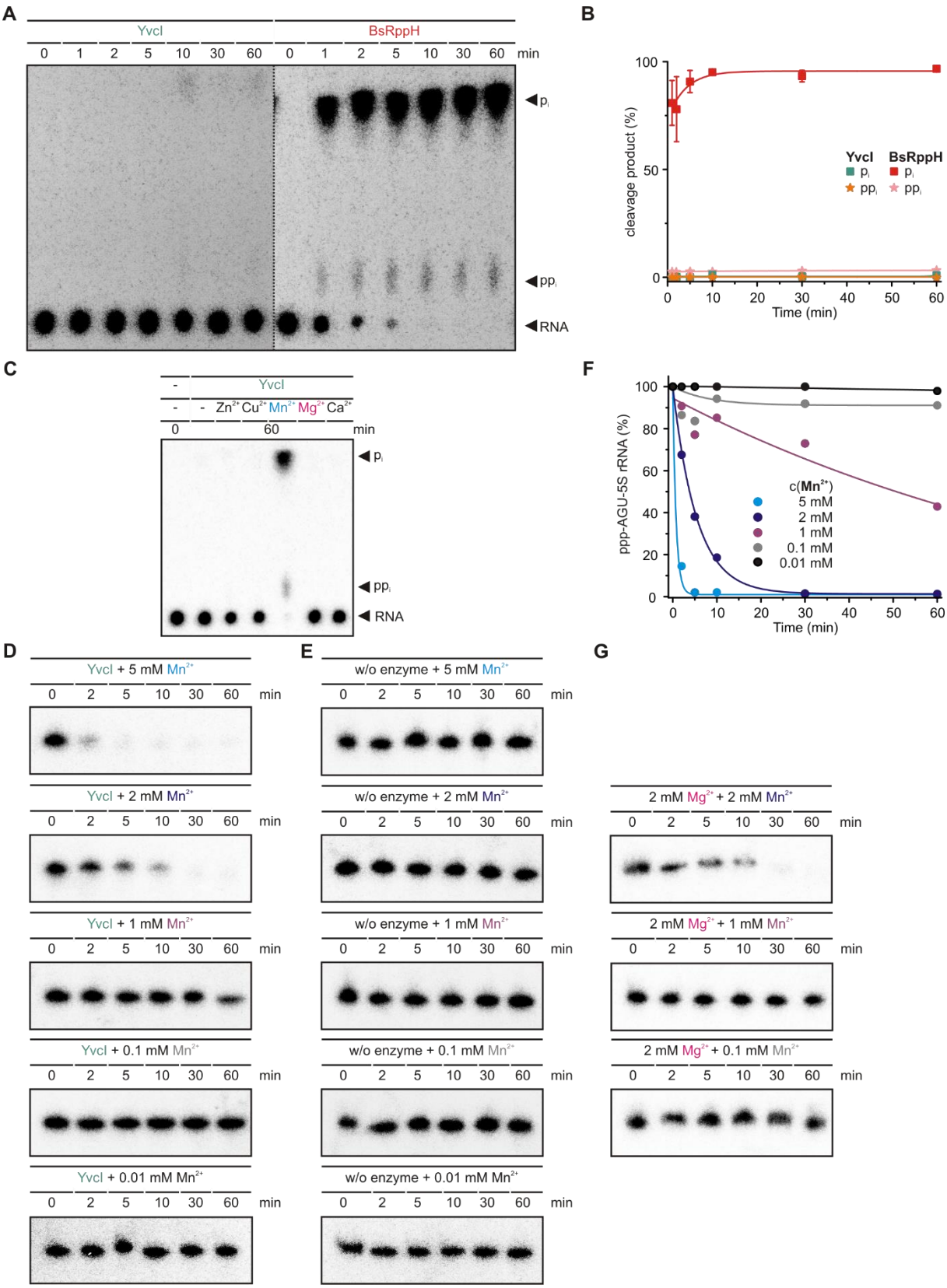

Figure S3. BsRppH is more active than YvcI, which is only active in presence of manganese. (**A**) Comparison of the RNA pyrophosphohydrolase activity of YvcI and BsRppH. ppp-RNA (AGU 5S rRNA, 5'-labeled, 0.5 µM) was incubated with YvcI or BsRppH (0.1 µM each) in 1× Mn-buffer (2 mM Mn^{2+}). The dotted line indicates where different parts of different plates were combined to facilitate interpretation. PEI-cellulose TLC, phosphor imaging of radioactivity. (**B**) Quantification of the cleavage products from (**A**), showing mean ± standard deviation of three independent experiments (technical replicates). (**C**) YvcI dephosphorylates ppp-RNA only in presence of manganese. ppp-RNA (AGU 5S rRNA, 5'-labeled, 0.5 µM) was incubated with YvcI (2 µM). Mn^{2+} was omitted (-) or exchanged by different divalent cations (Zn^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}; 2 mM).

Analysis as for (**A**). (**D**, **E**) YvcI requires 2 mM Mn^{2+} for efficient phosphate removal from ppp-RNA. ppp-RNA (AGU 5S rRNA, 5'-labeled, 0.5 µM) was incubated with (**D**) or without (**E**) YvcI (2 µM) in 1× Mn-buffer with 0, 0.01, 0.1, 1, 2, or 5 mM Mn^{2+}. 8% PAGE, phosphor imaging of radioactivity. (**F**) Quantification of the ppp-RNA from (**D**). (**G**) The presence of Mg^{2+} has no impact on the Mn^{2+}-dependent phosphate removal of ppp-RNA by YvcI. Description as for (**D**) with the indicated cation concentrations. 8% PAGE, phosphor imaging of radioactivity.

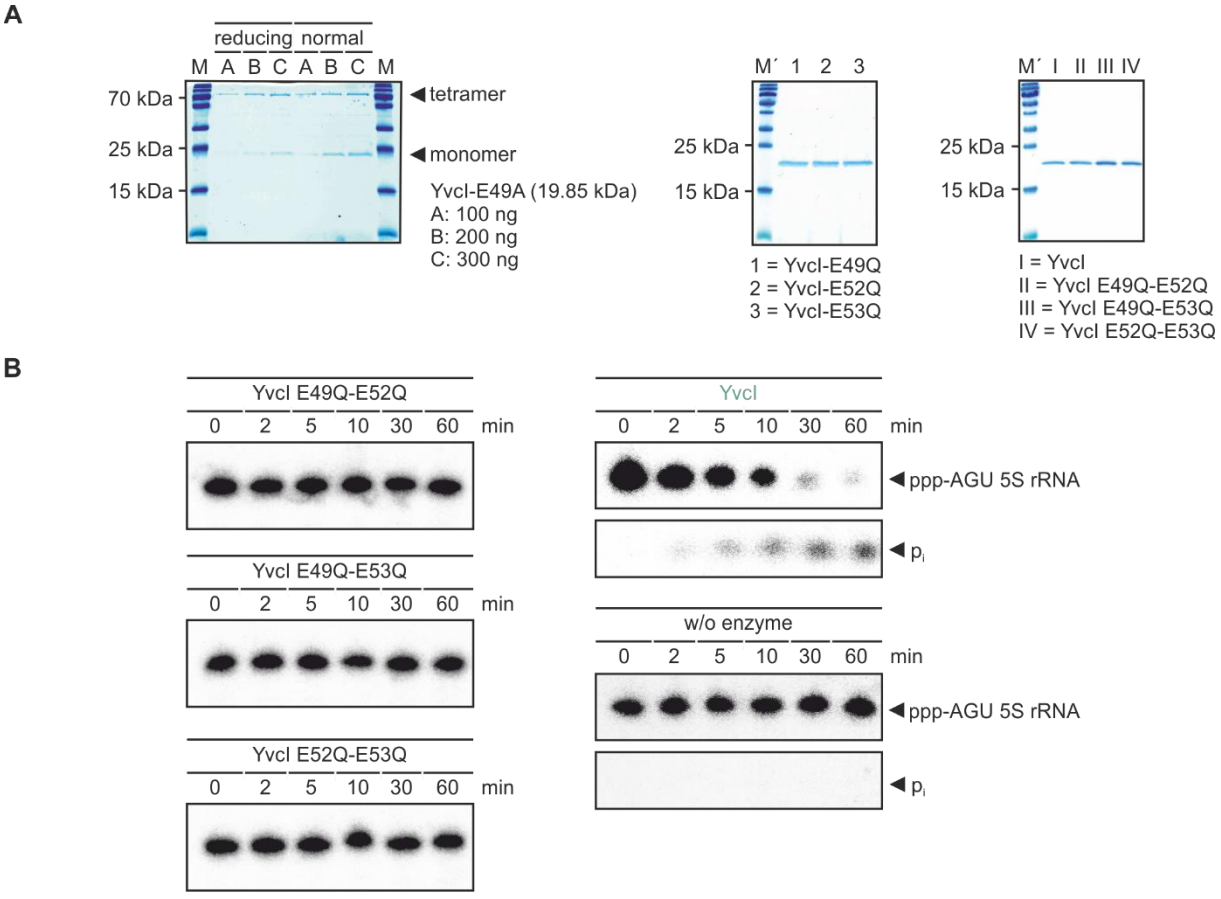

Figure S4. YvcI mutants with amino acid exchanges in the Nudix motive have no or a reduced RNA pyrophosphohydrolase activity. (**A**) Expression and purification of YvcI single and double mutants. The proteins were expressed in *E. coli* as His_6 fusions and purified by a combination of affinity and size exclusion chromatography. M, PageRuler Plus prestained protein ladder, M', PageRuler prestained protein ladder. 15% SDS-PAGE, Coomassie staining. (**B**) Two amino acid exchanges in the Nudix motif abolish the activity of YvcI. ppp-RNA (AGU 5S rRNA, 5'-labeled, 0.5 µM) was incubated with YvcI mutants, YvcI (2 µM, each) or without any enzyme in 1× Mn-buffer (2 mM Mn^{2+}). 8% PAGE, phosphor imaging of radioactivity.

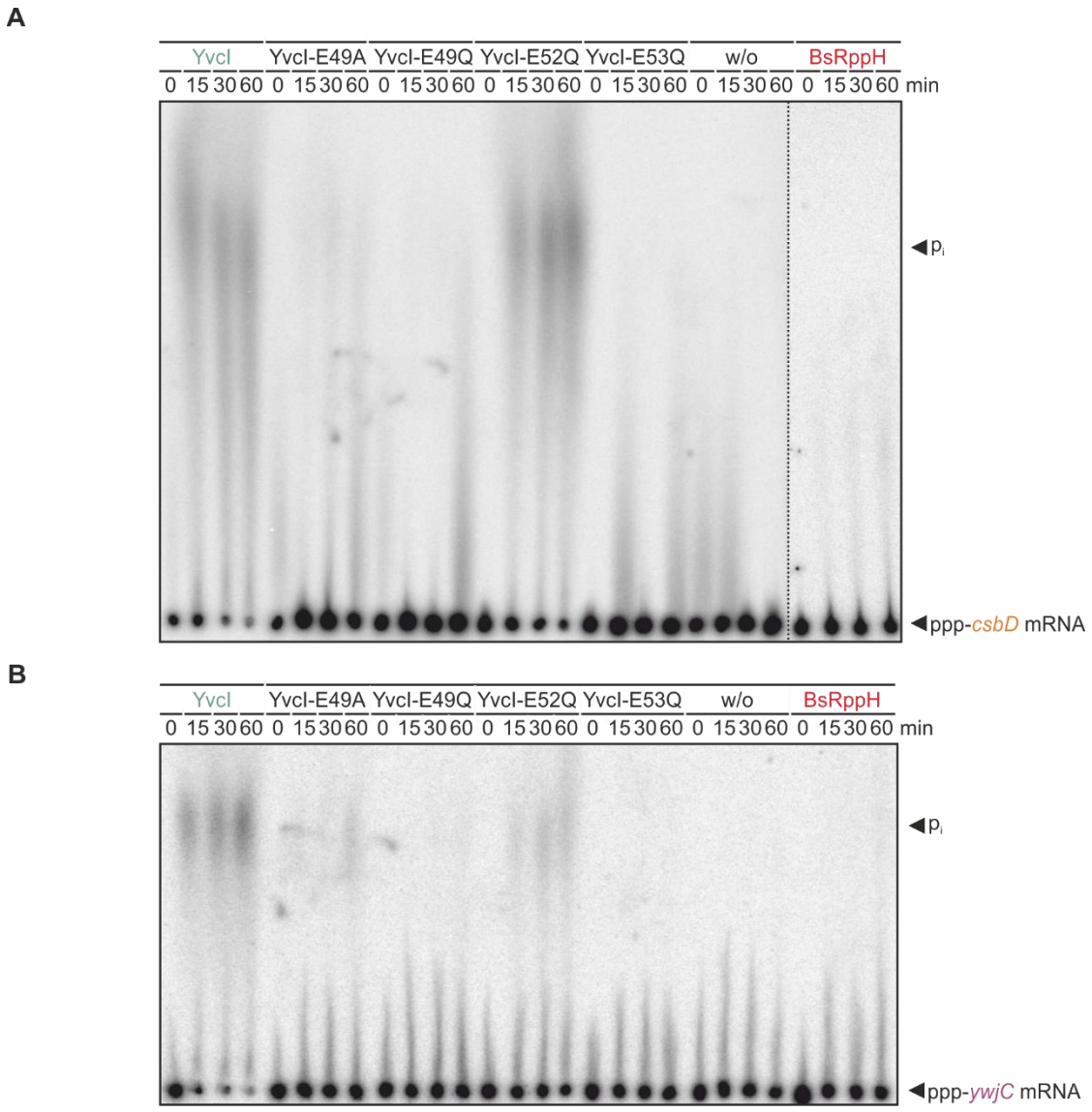

Figure S5. The Nudix motif of YvcI is responsible for phosphate removal from ppp-RNA. (**A**) YvcI mutants with amino acid exchanges in the Nudix motif were tested for their capability to remove phosphate from ppp-RNA. ppp-*csbD* mRNA (5'-labeled, 0.15 µM) was incubated with YvcI, YvcI mutants (0.6 µM each) or BsRppH (0.1 µM) in 1× Mn-buffer (2 mM Mn^{2+}) (1× Mg-buffer for BsRppH). The dotted line indicates where different parts of two plates were combined to facilitate interpretation. (**B**) Description as for (**A**), but ppp-*ywjC* mRNA was used as RNA substrate. PEI-cellulose TLC, phosphor imaging of radioactivity.

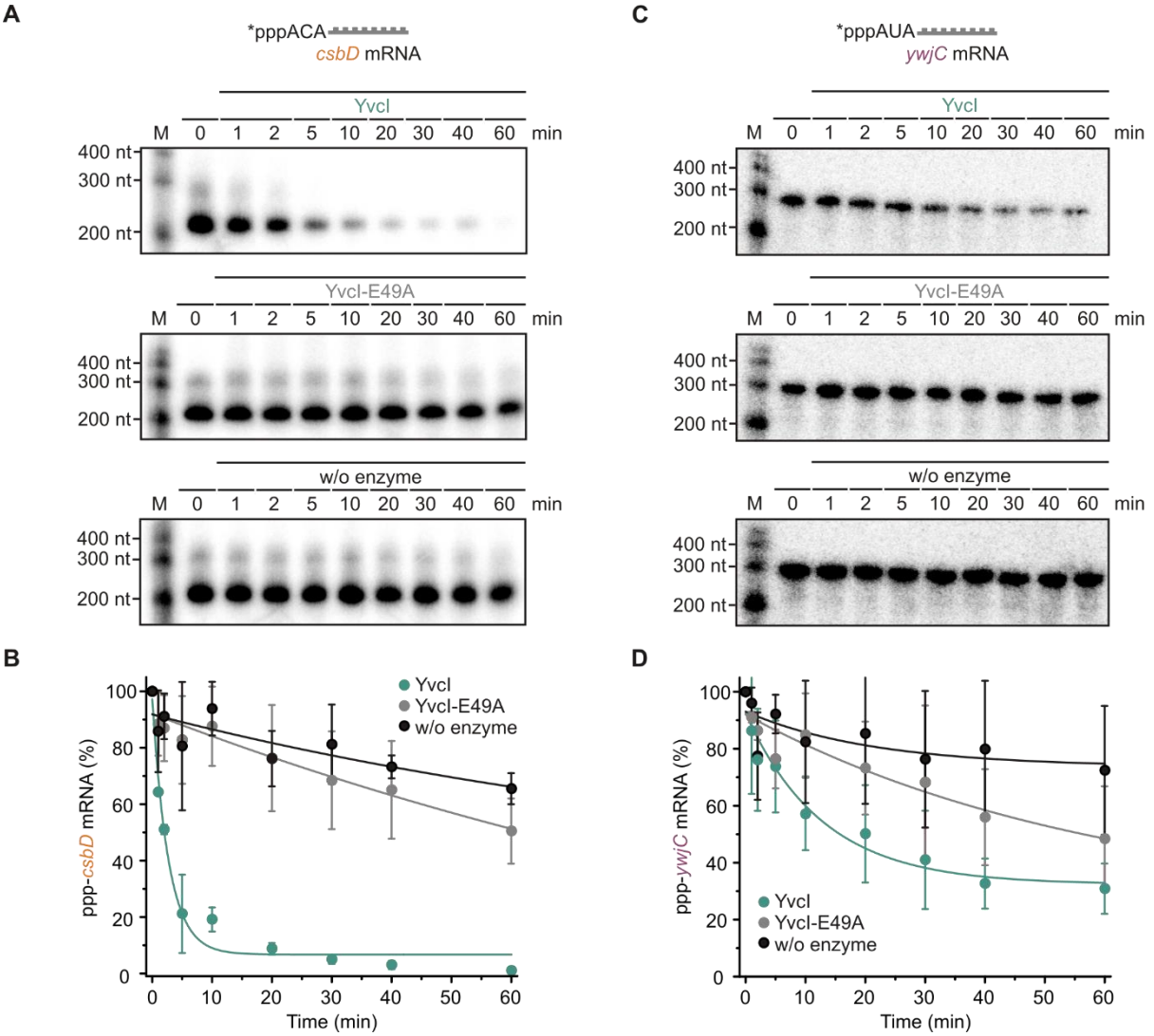

Figure S6. YvcI removes phosphate of ppp-*csbD* and ppp-*ywjC* mRNA with different efficiencies. (**A**) YvcI removes phosphate from ppp-*csbD* mRNA. Upper panel, scheme of ppp-*csbD* mRNA. An asterisk (*) indicates ^{32}P. ppp-*csbD* mRNA (5'-labeled, 0.25 µM) was incubated with YvcI (1 µM), YvcI-E49A (1 µM), or no enzyme, respectively, in 1× Mn-buffer (2 mM Mn^{2+}). 8% PAGE, phosphor imaging of radioactivity. (**B**) Quantification of the ppp-*csbD* mRNA from (**A**), showing mean ± standard deviation of three independent experiments (technical replicates). (**C**) YvcI removes phosphate from ppp-*ywjC* mRNA. Upper panel, scheme of ppp-*ywjC* mRNA. Description as for (**A**). 8% PAGE, phosphor imaging of radioactivity. (**D**) Quantification of the ppp-*ywjC* mRNA from (**C**). Statistics are as in (**B**).

SUPPORTING TABLES

Table S1. Bacterial strains used in this work.

Strain	Relevant genotype	Source/reference
Bacillus subtilis 1A1	*trpC2*	BGSC, Ohio
Escherichia coli BL21 Star (DE3)	*F⁻ ompT hsdS$_B$(r_B^- m_B^-) gal dcm (DE3)*	Thermo Scientific
Escherichia coli DH5α	*F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17($r_K^-m_K^+$), λ^-*	Thermo Scientific

Table S2. Primer used for cloning and mutation of various genes from *B. subtilis*. "p", phosphate, *Xba*I site, *Xho*I site, thrombin site.

Name	Sequence (5'-to-3' direction)	Used for
Fwd-YvcI	TTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAA TGACGTACTTGCAAAGAGTG	*yvcI* cloning
Rev-YvcI	AGGATTCTCGAGGCTGCCGCGCGGCACCAGTTTGATGTGCTGCGGG TCTA	
Fwd-BsRppH	TTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAA TGTACGAGTTTAAAGATTATTATCAGAA	*rppH* cloning
Rev-BsRppH	AGGATTCTCGAGGCTGCCGCGCGGCACCAGTTCAATCCATCCAGAT TCTTTC	
Fwd-NudF	TTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAA TGAAATCATTAGAAGAAAAAACAATTG	*nudF* cloning
Rev-NudF	AGGATTCTCGAGGCTGCCGCGCGGCACCAGTTTTTGTGCTTGGAGC GC	
Fwd-YmaB	TTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAA TGGGAAAAATGGACGAAATG	*ymaB* cloning
Rev-YmaB	AGGATTCTCGAGGCTGCCGCGCGGCACCAGTTCAAGAATATCAACA ACAAACTGTG	
Fwd-YjhB	TACTTCCAATCCAATGCAATAAAAGTGCAAACCAAATGGCT	*yjhB* cloning
Rev-YjhB	TTATCCACTTCCAATGTTATTAGTCAAATATCGTTTTCTTGTCCGG	
Fwd-MutT	TACTTCCAATCCAATGCATATACACAAGGTGCTTTTGTGATC	*mutT* cloning
Rev-MutT	TTATCCACTTCCAATGTTATTACTTGCCCAGTCTTCTTTTCAAC	
Fwd-YvcI-E49A	p-CGTACAGAGAAGAGACTGGTATC	*yvcI* mutation (E49A exchange)
Rev-YvcI-E49A	p-CTCTGATGACGGAGTCTCTGAC	
Fwd-YvcI-E49Q	p-CAGTACAGAGAAGAGACTGGTATC	*yvcI* mutation (E49Q exchange)
Rev-YvcI-E49Q	p-TCTGATGACGGAGTCTCTGAC	
Fwd-YvcI-E52Q	p-CAAGAGACTGGTATCTATATCATAAATCC	*yvcI* mutation (E52Q exchange)
Rev-YvcI-E52Q	p-TCTGTACTCTCTGATGACGG	
Fwd-YvcI-E53Q	p-CAGACTGGTATCTATATCATAAATCC	*yvcI* mutation (E53Q exchange)
Rev-YvcI-E53Q	p-TTCTCTGTACTCTCTGATGAC	
Fwd-YvcI-E49Q-E52Q	p-CAAGAGACTGGTATCTATATCATAAATCC	*yvcI* mutation (E49Q, E52Q exchange); pET-28a-YvcI-E49Q (template)
Rev-YvcI-E49Q-E52Q	p-TCTGTACTGTCTGATGACG	
Fwd-YvcI-E49Q-E52/3Q	p-CAGACTGGTATCTATATCATAAATCCTC	*yvcI* mutation (E49Q, E52/3Q exchange); pET-28a-YvcI-E49Q (template) pET-28a-YvcI-E52Q (template)
Rev-YvcI-E49Q-E53Q	p-TTCTCTGTACTGTCTGATGAC	
Rev-YvcI-E52Q-E53Q	p-TTGTCTGTACTCTCTGATGACG	

Table S3. Primers used for the amplification of regions of interest from genomic DNA. The resulting PCR products served as templates for *in vitro* transcription reactions. For the preparation of GA(CU)$_{13}$ RNA and AG(CU)$_{13}$ RNA, the respective oligo nucleotides were annealed without PCR amplification. T7 (Φ2.5) promotor marked in bold, T7 (Φ6.5) promotor marked in bold italics. Appended nucleotides in blue and magenta, respectively. "m", 2' O-methyl RNA base.

ID	Sequence (5'-to-3' direction)	Used in/for
Fwd-gapB 75mer	TC**TAATACGACTCACTATT**AGATAGTAACAAATTAGGATGG	*gapB* 75mer
Rev-gapB 75mer	mAmACCCGTTGATCGCTACTTTTAC	
Fwd2-5S rRNA-A	AT**TAATACGACTCACTATT**ATCTGGTGATGATGGCGAAGAGG	combination with Rev-5S rRNA
Fwd2-5S rRNA-AGU	AT**TAATACGACTCACTATT**AGTTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-AGU	AT**TAATACGACTCACTATT**AGTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-ACU	AT**TAATACGACTCACTATT**ACTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-AAU	AT**TAATACGACTCACTATT**AATGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-AUU	AT**TAATACGACTCACTATT**ATTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA blunt end	AT***TAATACGACTCACTATA***GTCTGGTGATGATGGCGAAGAGG	combination with Rev-5S rRNA-+1nt 3'
Fwd-5S rRNA-A	AT**TAATACGACTCACTATT**AGTCTGGTGATGATGGCGAAGAGG	combination with Rev-5S rRNA
Fwd-5S rRNA-AG	AT**TAATACGACTCACTATT**AGGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-AGUG	AT**TAATACGACTCACTATT**AGTGGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-GGU	AT***TAATACGACTCACTATA***GGTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-GAU	AT***TAATACGACTCACTATA***GATGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-GCU	AT***TAATACGACTCACTATA***GCTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-GUU	AT***TAATACGACTCACTATA***GTTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-G	AT**TAATACGACTCACTATT**GGTCTGGTGATGATGGCGAAGAGG	combination with Rev-5S rRNA
Fwd-5S rRNA-GG	AT**TAATACGACTCACTATT**GGGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-GGU	AT**TAATACGACTCACTATT**GGTGTCTGGTGATGATGGCGAAGAGG	
Fwd-5S rRNA-GGUG	AT**TAATACGACTCACTATT**GGTGGTCTGGTGATGATGGCGAAGAGG	
Rev-5S rRNA	GCCTGGCGATGTCCTACTCTC	
Rev-5S rRNA-+1nt 3'	GGCCTGGCGATGTCCTACTCTC	
Fwd-GA(CU)$_{13}$	TA***TAATACGACTCACTATA***GACTCTCTCTCTCTCTCTCTCTCTCT	GA(CU)$_{13}$ RNA
Rev-GA(CU)$_{13}$	AGAGAGAGAGAGAGAGAGAGAGAGTCTATAGTGAGTCGTATTATA	
Fwd-AG(CU)$_{13}$	TA**TAATACGACTCACTATT**AGCTCTCTCTCTCTCTCTCTCTCT	AG(CU)$_{13}$ RNA
Rev-AG(CU)$_{13}$	AGAGAGAGAGAGAGAGAGAGAGAGCTAATAGTGAGTCGTATTATA	
Fwd-veg	TA**TAATACGACTCACTATT**AGTGAGGTGGATGCAATGGC	*veg* mRNA
Rev-veg	AGGGCAAAACAAAAAGCAGTAAAC	
Fwd-csbD	TA**TAATACGACTCACTATT**ACACACATAATTGAGAGGG	*csbD* mRNA
Rev-csbD	TTAATCTTTATCTGAAAATTTATCC	
Fwd-ywjC	TA**TAATACGACTCACTATT**ATAAGACAACAATCGATGG AGG	*ywjC* mRNA
Rev-ywjC	TTAGAGCGCTTCTCTCTCAGC	

Table S4. Plasmids used in this work.

Name	Relevant fragment	Used for	Reference/Supplier
pET His6 MBP TEV LIC (2M-T)	-	ligation independent cloning (LIC)	Addgene
pET-28a(+)	-	general cloning	Novagen
pET-28a-YvcI	*yvcI*	expression of YvcI	This work
pET-28a-YvcI-E49A	*yvcI*-E49A	expression of YvcI-E49A	This work
pET-28a-YvcI-E49Q	*yvcI*-E49Q	expression of YvcI-E49Q	This work
pET-28a-YvcI-E52Q	*yvcI*-E52Q	expression of YvcI-E52Q	This work
pET-28a-YvcI-E53Q	*yvcI*-E53Q	expression of YvcI-E53Q	This work
pET-28a-YvcI-E49Q-E52Q	*yvcI*-E49Q-E52Q	expression of YvcI-E49Q-E52Q	This work
pET-28a-YvcI-E49Q-E53Q	*yvcI*-E49Q-E53Q	expression of YvcI-E49Q-E53Q	This work
pET-28a-YvcI-E52Q-E53Q	*yvcI*-E52Q-E53Q	expression of YvcI-E52Q-E53Q	This work
pET-28a-BsRppH	*rppH*	expression of BsRppH	(4)
pET-28a-NudF	*nudF*	expression of NudF	This work
pET-28a-YmaB	*ymaB*	expression of YmaB	This work
2M-T-YjhB	*yjhB*	expression of YjhB	This work
2M-T-MutT	*mutT*	expression of MutT	This work

SUPPLEMENTARY REFERENCES

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