Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

Peptide Nucleic Acid Mediated Inhibition of the Bacterial Signal Recognition Particle

Sudipta Ghosh,[a] Snehlata Saini[b] and Ishu Saraogi[a,b]

[a.] *Department of Chemistry*
Indian Institute of Science Education and Research Bhopal
Bhopal Bypass Road, Bhauri, Bhopal 462066, MP, India
E-mail: ishu@iiserb.ac.in
[b.] *Department of Biological Sciences*
Indian Institute of Science Education and Research Bhopal
Bhopal Bypass Road, Bhauri, Bhopal 462066, MP, India

Table of Contents

Supplementary figures:

[#] provided by Panagene Inc. (Korea)

Experimental methods and materials:

Supplementary figures

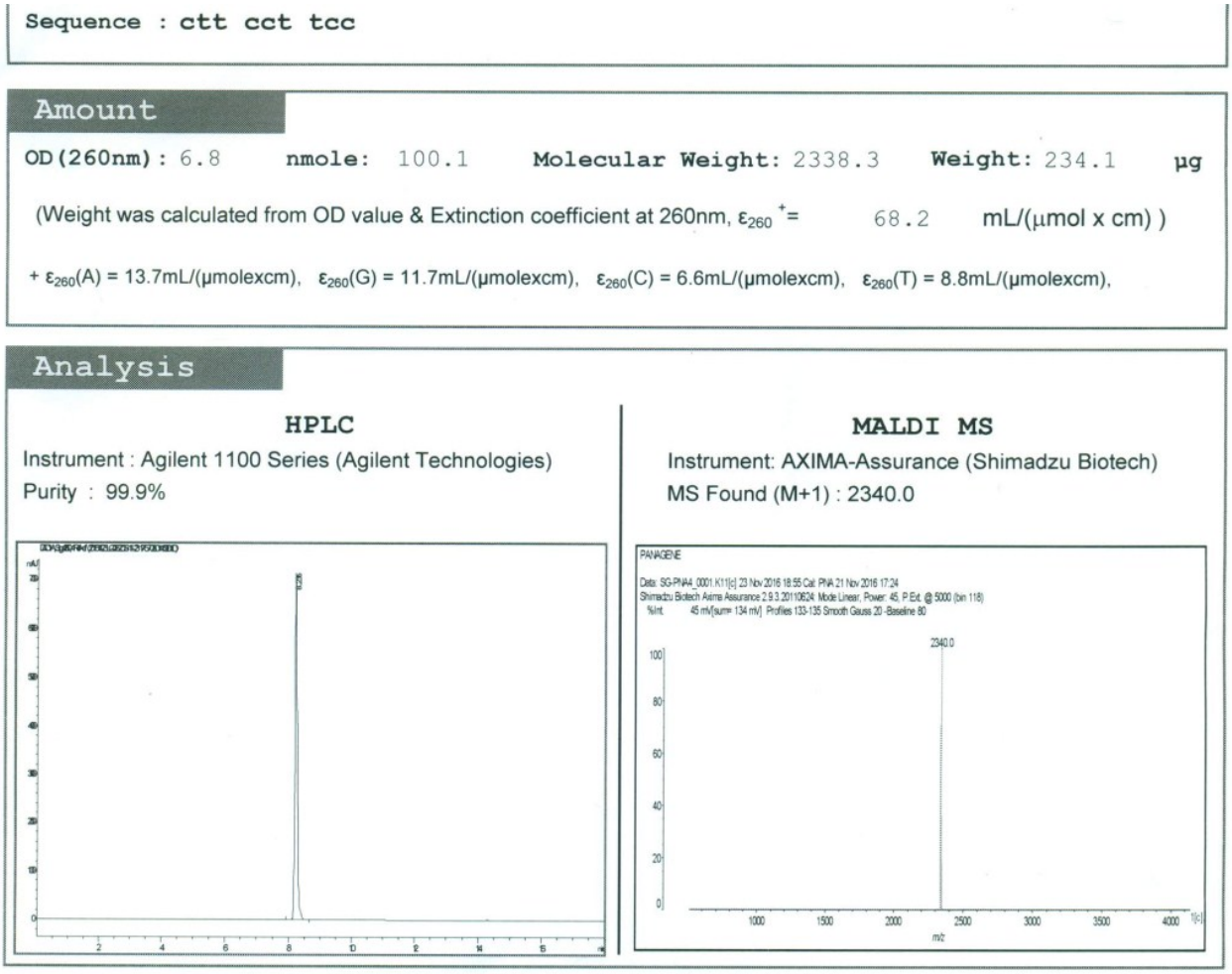


Figure S1. PNA1 purity and characterization data.

Sequence : cct gac ct

Amount

OD(260nm): 13.9 **nmole:** 200.1 **Molecular Weight:** 2121.0 **Weight:** 424.3 **µg**

(Weight was calculated from OD value & Extinction coefficient at 260nm, ε_{260} $^{+}$= 69.4 mL/(µmol x cm))

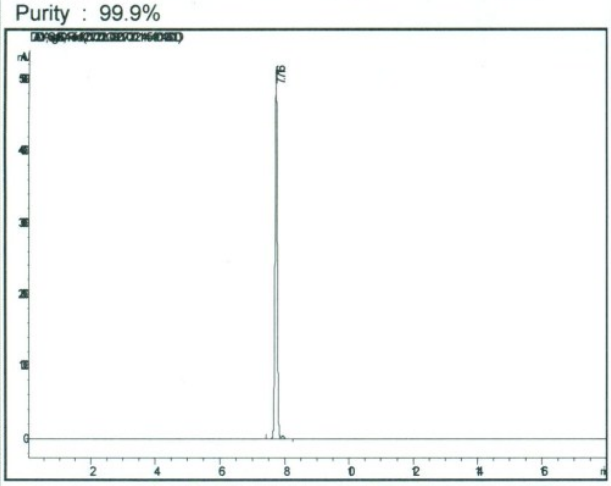
+ ε_{260}(a) = 13.7mL/(µmolexcm), ε_{260}(g) = 11.7mL/(µmolexcm), ε_{260}(c) = 6.6mL/(µmolexcm), ε_{260}(t) = 8.8mL/(µmolexcm),

Analysis

HPLC

Instrument : Agilent 1100 Series (Agilent Technologies)

Purity : 99.9%



MALDI MS

Instrument: AXIMA-Assurance (Shimadzu Biotech)

MS Found (M+1) : 2122.5

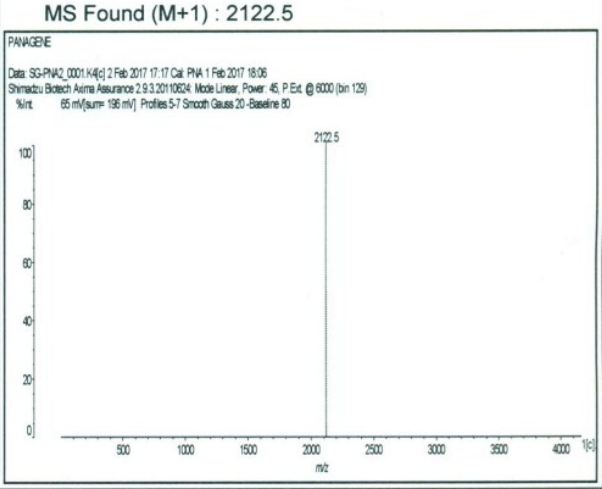


Figure S2. PNA2 purity and characterization data.

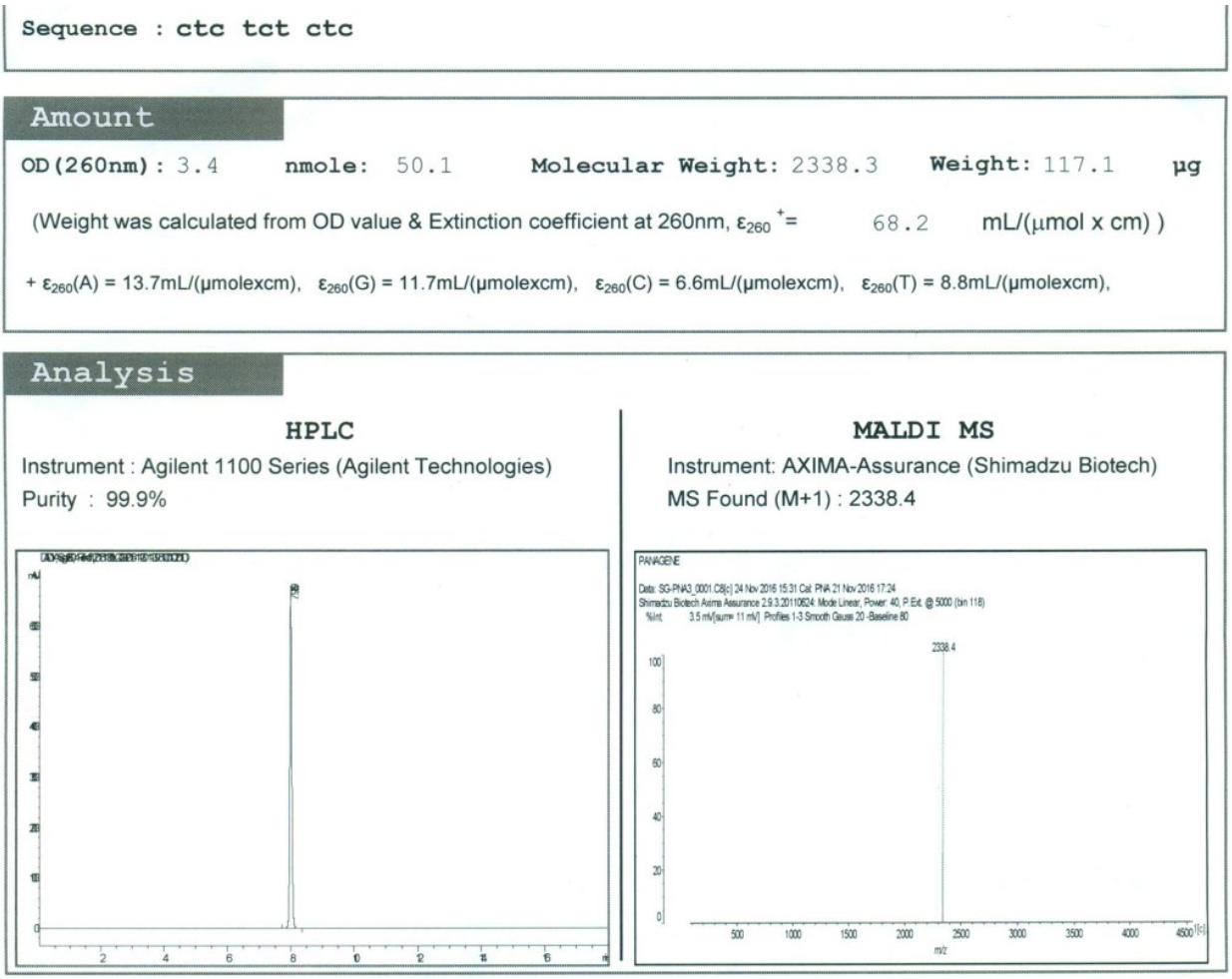


Figure S3. PNA3 purity and characterization data.

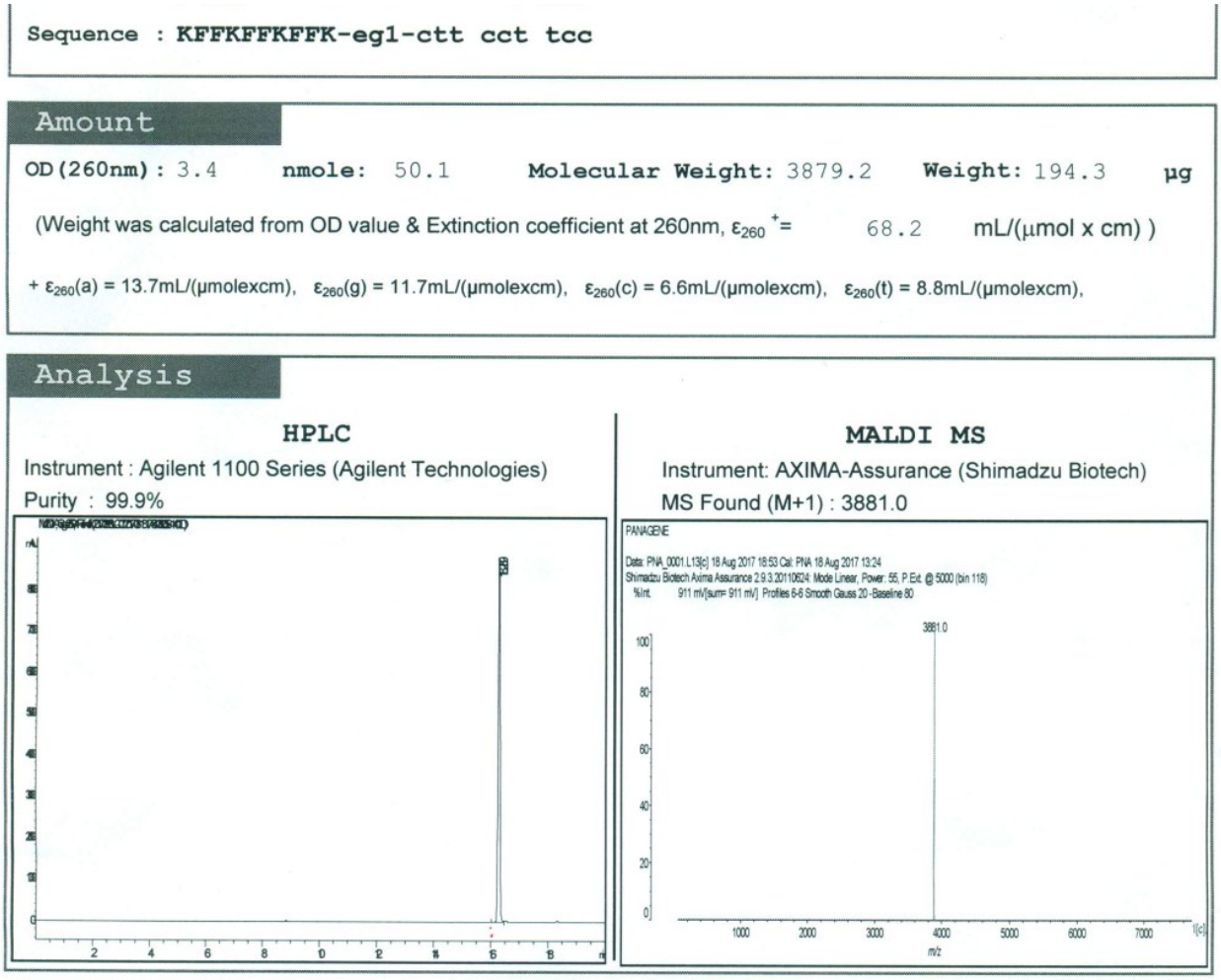


Figure S4a. PNA1-CPP purity and characterization data.

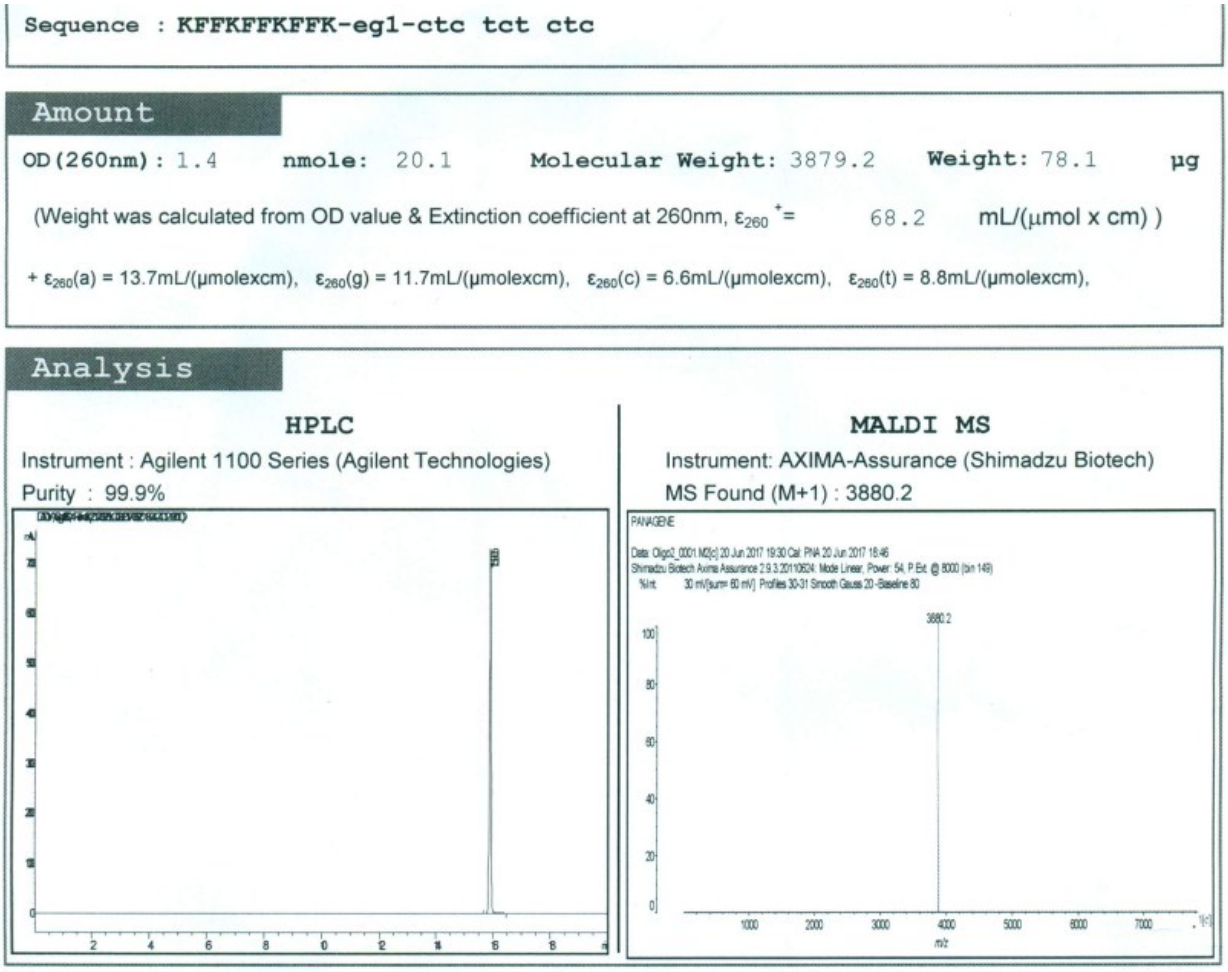


Figure S4b. PNA3-CPP purity and characterization data.

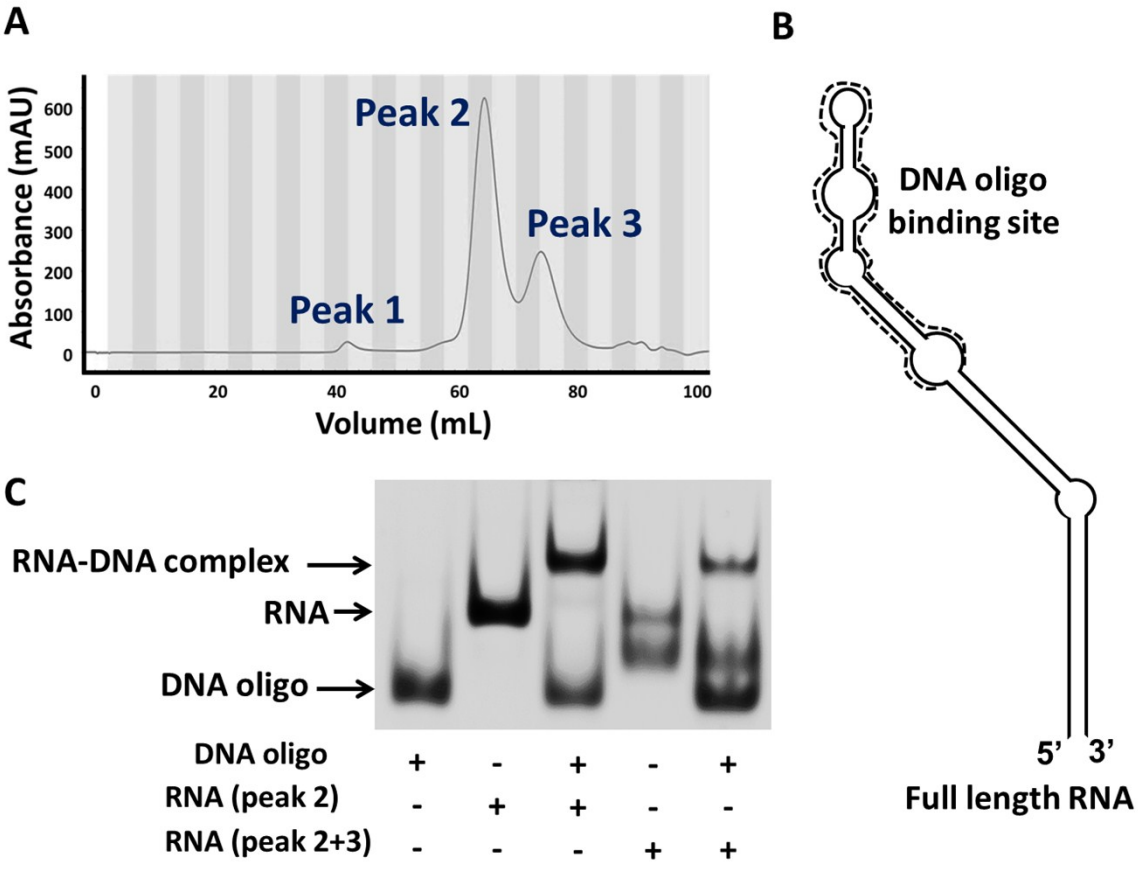


Figure S5. Purification and identification of 4.5S RNA overexpressed from *E. coli* cells. (A) Size exclusion chromatogram of total RNA isolated from *E. coli* DH5α cells over-expressing 4.5S RNA. (B) A schematic representation of the complementary 50-mer DNA oligo (5'GTCATCTGCCTTGGCTGCTTCCTTCCGGACCTGACCTGGTAAACAGAGTA3') binding to target 4.5S RNA (dotted line). (C) EMSA experiment with the complementary DNA oligo to identify the 4.5S RNA peak in the chromatogram.

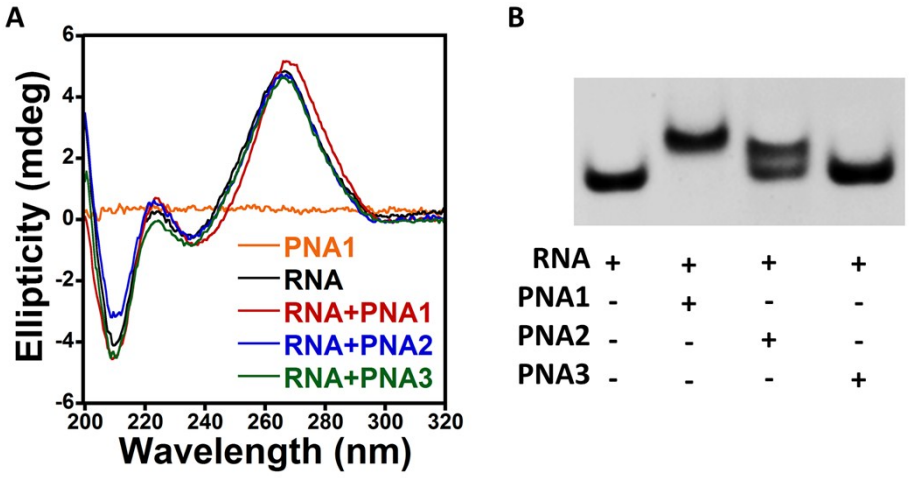


Figure S6. Interaction of designed PNA oligomers with full-length 4.5S RNA. (A) Circular dichroism analysis of full-length wild type RNA (0.5µM), in presence and absence of PNA oligomers (5µM). (B) EMSA analysis of full-length 4.5S RNA (2µM) and PNA (10µM) interaction analyzed on a 7% native PAGE.

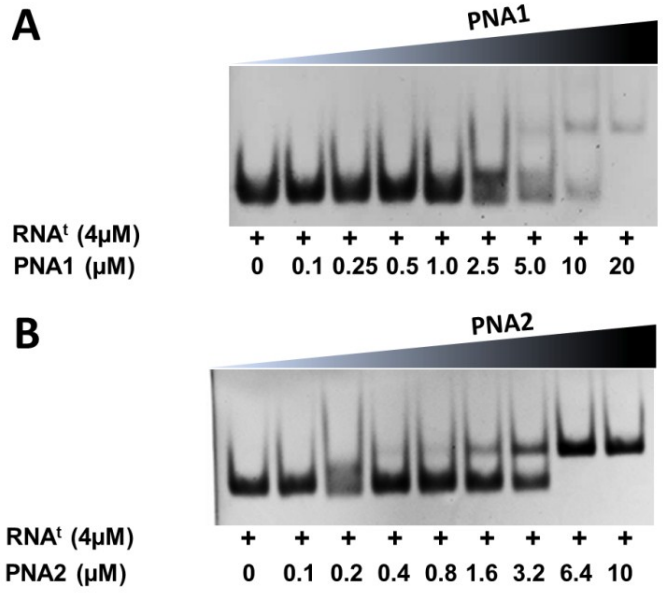


Figure S7. Dose-dependent interaction of PNA1 and PNA2 with RNA^t. Indicated concentrations of (A) PNA1 or (B) PNA2 were used for complex formation with RNA^t.

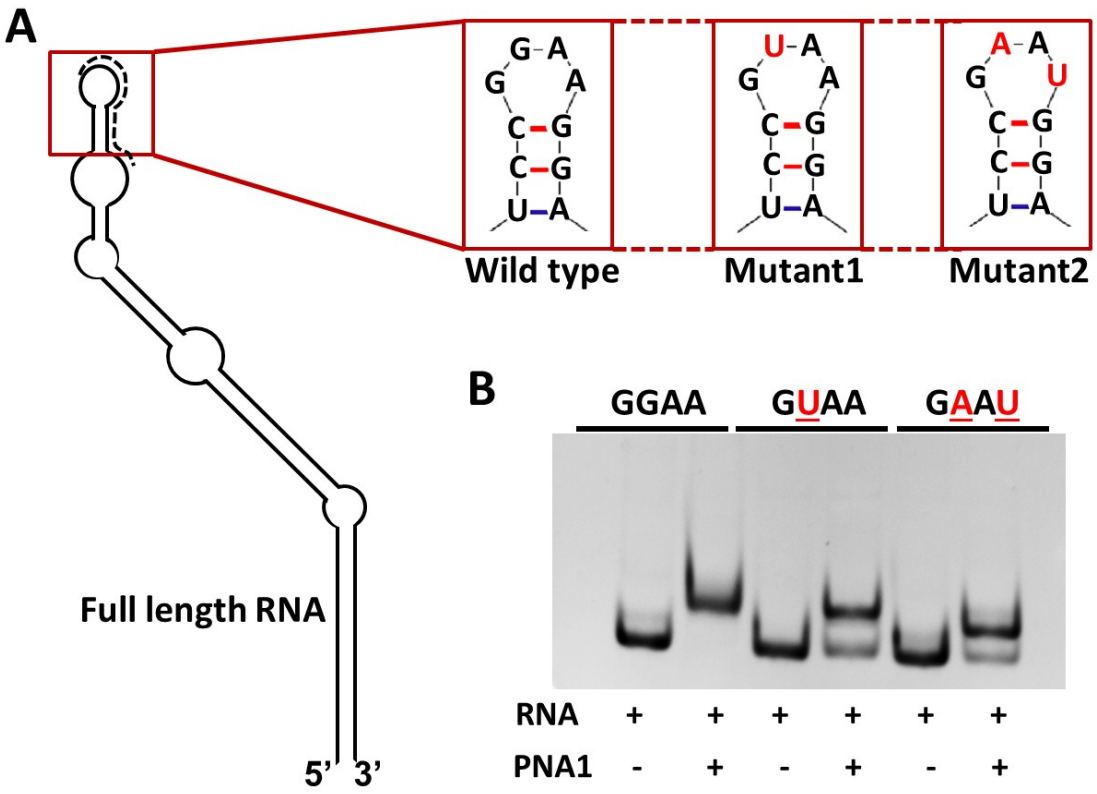


Figure S8. Specificity of 4.5S RNA and PNA interaction. (A) The PNA1 target site (black dotted line) in the tetraloop region of full-length 4.5S RNA was mutated to generate two RNA mutants with one (mutant1) and two base mismatches (mutant2) for PNA1. (B) EMSA gel showing the binding of wild type and mutant 4.5S RNAs (2µM each), containing 1 or 2 mismatches (underlined), to PNA1 (10µM).

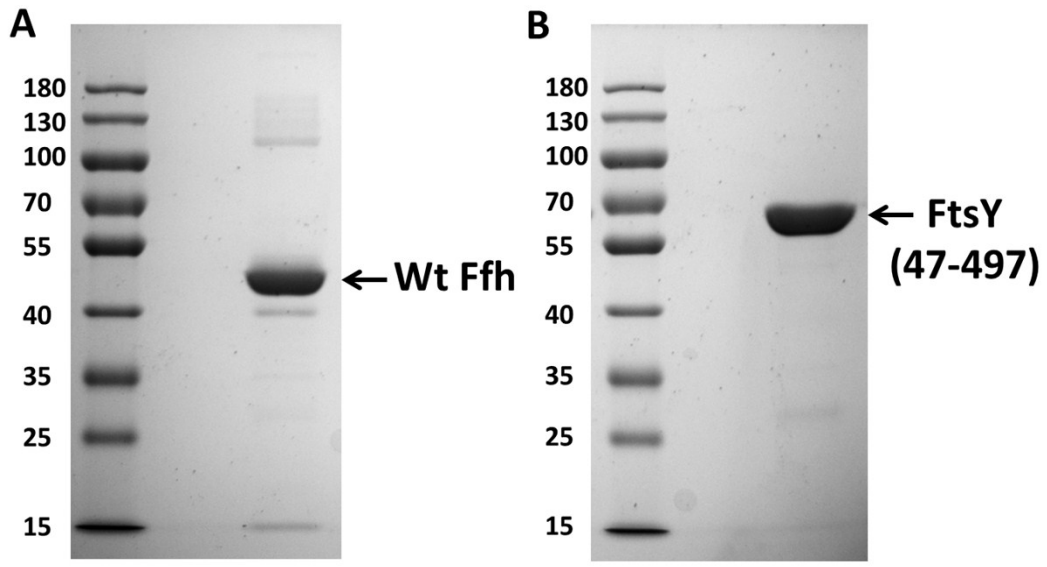


Figure S9. SDS-PAGE analysis of the purified Ffh and FtsY proteins from *E. coli*.

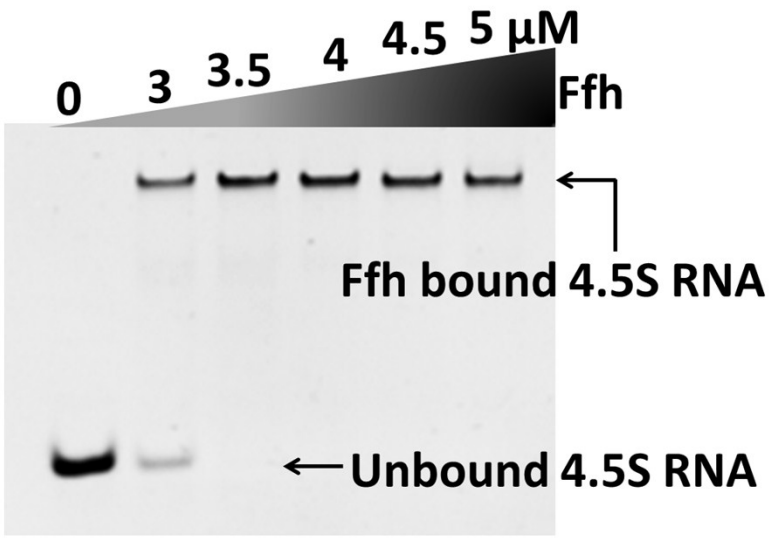


Figure S10. EMSA analysis of Ffh and full-length RNA interaction. A fixed concentration of RNA (2µM) was treated with specified concentrations of Ffh.

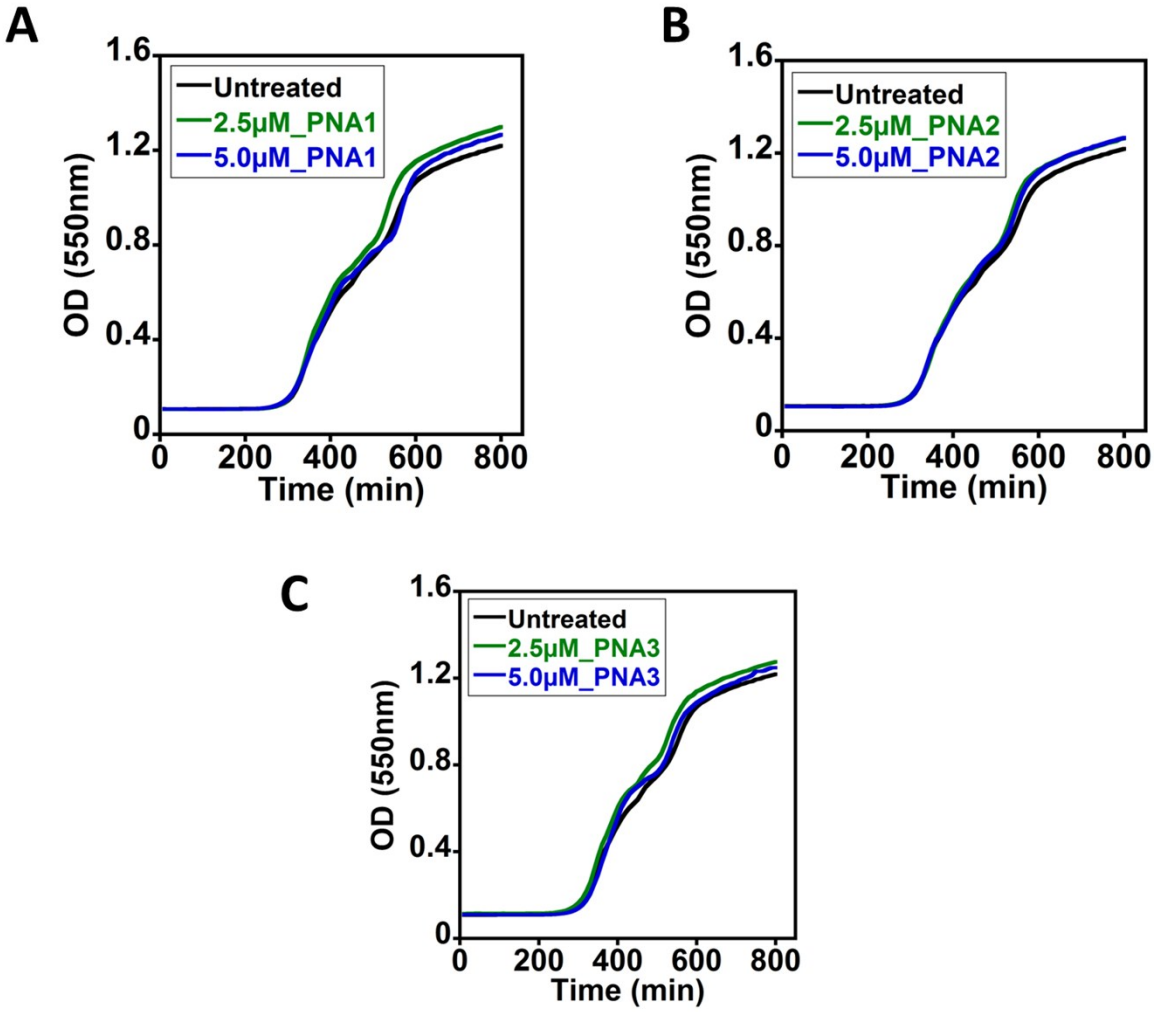


Figure S11. Effect of unmodified PNAs on *E. coli* AS19 cell growth.

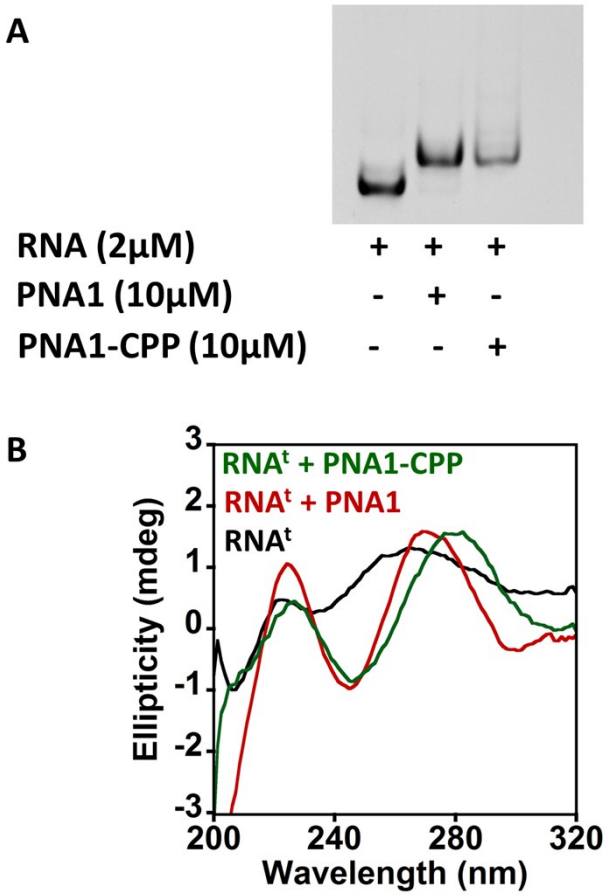


Figure S12. A comparative analysis of PNA1 and PNA1-CPP interaction with 4.5S RNA. (A) EMSA and (B) CD experiments were performed as described.

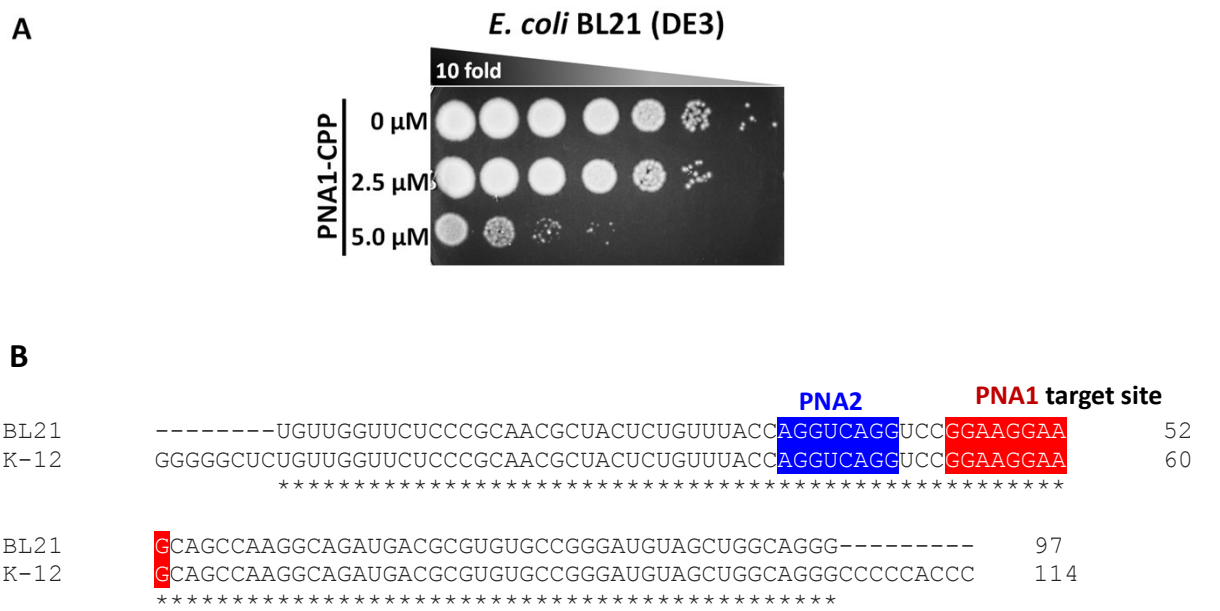


Figure S13. Inhibitory effect of PNA1-CPP on (A) *E. coli* BL21 (DE3) cells. 10-fold dilution refers to the serial dilution of bacterial samples from left to right in each row. (B) Alignment of 4.5S RNA sequences of *E. coli* B-type (BL21) and K-12 (AS19).

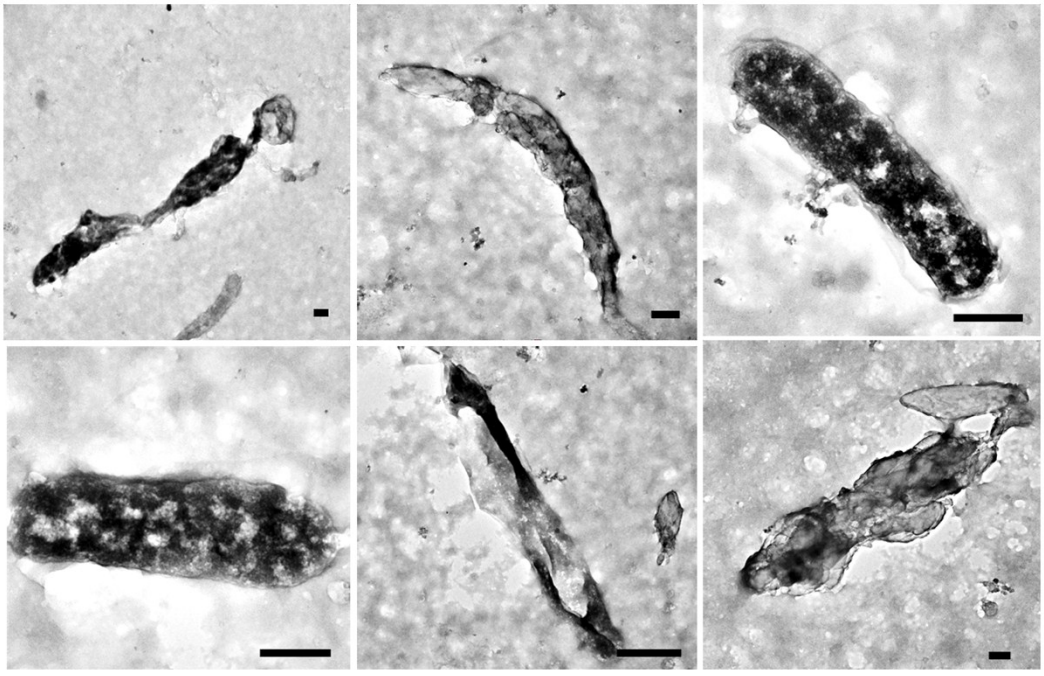


Figure S14. TEM images showing 3µM PNA1-CPP treated AS19* cells. Scale bar = 500nm.

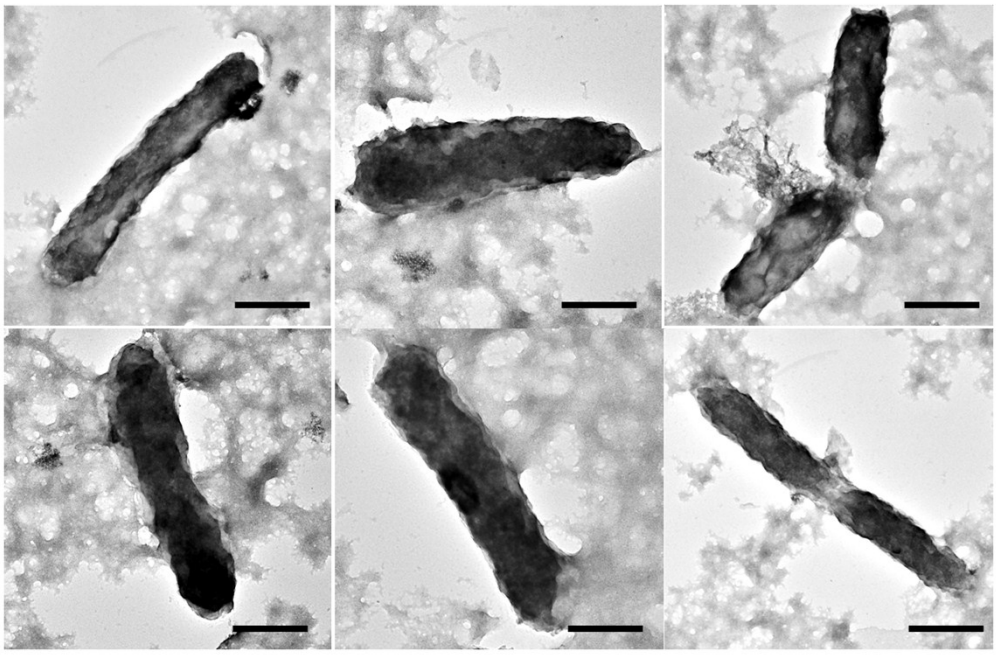


Figure S15. TEM images of AS19* cells treated with 3µM PNA1-CPP and 1mM IPTG. Scale bar = 500nm.

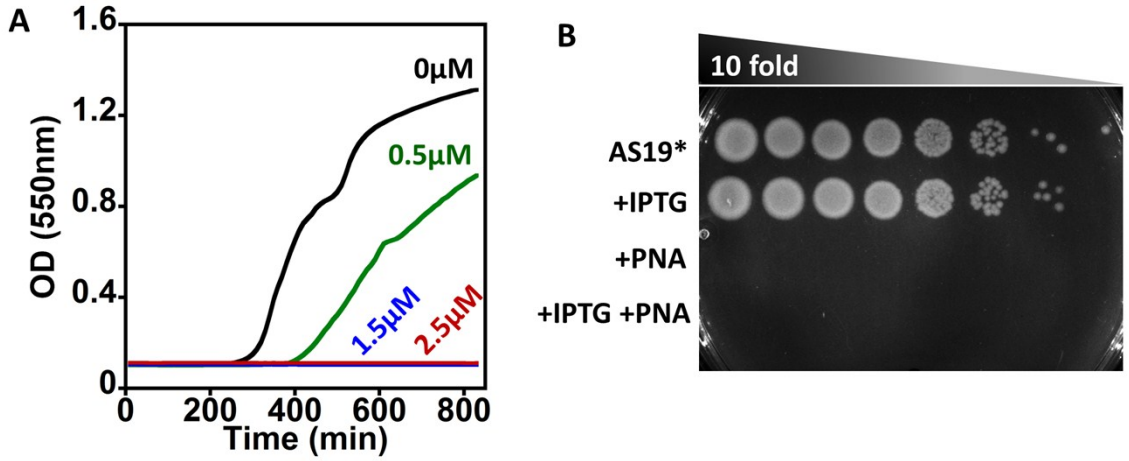


Figure S16. The effect of PNA3-CPP on AS19 or AS19* cells. A) The dose-dependent effect of PNA3-CPP on *E. coli* AS19 cell growth in liquid MHB culture. B) The inhibition of AS19* cells by PNA3-CPP (3 µM) in a spot assay. For this assay, 10 fold serially diluted bacterial samples from each treatment were spotted on agar plate. No relief of PNA-mediated inhibition of AS19* cell-growth was observed upon IPTG induced overexpression of 4.5S RNA (row 4).

Experimental methods and materials:

Table S1: Alignment of SRP RNA sequences from several organisms (Source: Signal Recognition Particle Database, SRPDB).[1]

	Organism	SRP RNA sequence alignment (5' to 3')
	H. sapiens	CGGCCCAGGUCGGAAACGGAGC
	M. jannaschii	CCCGCCAGGCCCGGAAGGGAGC
	M. tuberculosis	- CCUUCCAGCCCUGGGGGAG- -
Gram +ve	*S. aureus*	CAUGUCAGGUCCUGACGGAAGC
Gram +ve	*B. subtilis*	CAUGUCAGGUCCGGAAGGAAGC
Gram +ve	*L. monocytogenes*	CAUGUCAGGUCCGGAAGGAAGC
Gram -ve	*H. pylori*	CGCUUCAGGGUAGGAAUACAGC
Gram -ve	*P. aeruginosa*	CUGGUCAGGUCCGGAAGGAAGC
Gram -ve	*V. cholerae*	CUGGUCAGAUCCGGAAGGAAGC
Gram -ve	*S. marcescens*	UCGGUCAGGUCCGGAAGGAAGC
Gram -ve	*K. pneumoniae*	CAGGUCAGGUCCGGAAGGAAGC
Gram -ve	*S. typhimurium*	CAGGUCAGGUCCGGAAGGAAGC
Gram -ve	*S. dysenteriae*	CAGGUCAGGUCCGGAAGGAAGC
Gram -ve	*E. coli*	CAGGUCAGGUCCGGAAGGAAGC
		PNA2 PNA1

Red and blue regions indicate the PNA1 and PNA2 target sites respectively.

Table S2: Different nucleic acid oligomers used in the study.

Name (length)	Sequences (5' to 3')
Full length 4.5S RNA (114-mer)	GGGGGCUCUGUUGGUUCUCCCGCAACGCUACUCUGUUUACCAGGUCAGG UCCGGAAGGAAGCAGCCAAGGCAGAUGACGCGUGUGCCGGGAUGUAGCU GGCAGGGCCCCCACCC
RNA^{t} (26-mer)	AGGUCAGGUCCGGAAGGAAGCAGCCA
PNA1 (9-mer)[a]	CTTCCTTCC
PNA2 (8-mer)[a]	CCTGACCT
PNA3 (9-mer)[a]	CTCTCTCTC
PNA1-CPP[a]	KFFKFFKFFK-eg1-CTTCCTTCC
PNA3-CPP[a]	KFFKFFKFFK-eg1-CTCTCTCTC

[a] The purity and characterization data are given in supplementary figures S1-S4.

Table S3: The cellular targets and MIC values of some reported PNA scaffolds showing antibacterial effect in *E. coli*.

S. No.	PNA target site	MIC value	Bacterial strain	References
A.	TEM-1 β-lactamase gene	2.5-25 µM	*E. coli* DH5α	2
B.	23S ribosomal RNA	15 µM	*E. coli*	3
C.	*acpP*, *FtsZ* and *murA* genes	1.2- >10 µM	*E. coli*	4

Expression and purification of 4.5S RNA

The wild type 4.5S RNA (114-mer) was overexpressed in *E. coli* DH5α cells (Sigma-Aldrich) from pSN1 vector, with carbenicillin (100μg/mL) as the selection marker. The culture was grown to an OD_{600} of 0.5, and was induced with IPTG (1mM) for 10 hrs at 37°C. The total RNA from the induced cells was isolated as described earlier.[5] 4.5S RNA mutants, containing a single (GUAA) or double mutation (GAAU), were also purified as described above. The 26-mer RNA fragment (RNA^t) was ordered from Sigma-Aldrich, USA.

Characterization of 4.5S RNA using electrophoretic mobility shift assay (EMSA)

The identity of purified 4.5S RNA was verified using an EMSA assay. For this experiment, a 50-mer DNA oligonucleotide complementary to the 4.5S RNA sequence was designed and procured from IDT. The samples were dissolved in appropriate volume of binding buffer (20mM HEPES, 150mM NaCl, 0.5mM EDTA, pH 7.0) to get the desired stock concentration. The RNA samples (2μM), with or without the DNA oligo (10μM), were heated to 95°C for 5 min for complete denaturation, and then cooled down to room temperature. Samples were analyzed by 7% native PAGE (acrylamide/bisacrylamide 19:1, w/w) in 1X TBE buffer (pH 8.3) for 45 min at 70V. Electrophoresis was performed at room temperature, and gels were visualized using ethidium bromide staining on a UV gel doc system (G:Box Chemi-XRQ, Syngene, USA).

PNA oligomers used in the study

PNA oligomers (PNA1 and PNA2) were designed to bind to selected target sequences in domain IV of 4.5S RNA via Watson-Crick base pairing. PNA oligomer (PNA3), which was not complementary to the 4.5S RNA, served as a mis-match control. A cell penetrating peptide sequence (KFFKFFKFFK) was conjugated with PNA1 and PNA3 to evaluate the effect of PNA on bacterial growth. All PNA oligomers and their CPP conjugates were obtained from Panagene Inc. (Korea), and their purity and characterization data are given in supplementary figures S1-S4.

Expression and purification of Ffh and FtsY (47-497)

Ffh and FtsY (47-497) were overexpressed and purified as described by Peluso *et al.*[5] Briefly, Ffh was overexpressed from a pET vector in *E. coli* BL-21(DE3) (Sigma-Aldrich). Cells were grown in LB medium supplemented with ampicillin (100μg/ml) at 37°C until the absorbance at 600nm reached 0.6. Protein expression was induced with 1mM IPTG and the cells were grown at 37°C for an additional 3 hrs. Cells were harvested by centrifugation and resuspended in buffer A (20mM HEPES, pH 8.0, 2mM EDTA, 250mM NaCl, 2mM DTT, 1mM PMSF and 10% glycerol). Cells were lysed by sonication, and the protein was purified using SP-Sepharose fast flow resin, followed by gel-filtration using a Superose-12 column.

FtsY was overexpressed from pET vector in *E. coli* BL-21(DE3) (Sigma-Aldrich). Cells were grown in LB medium supplemented with 0.4% dextrose and ampicillin (100μg/ml) at 37°C until the absorbance at 600nm reached 0.6. Protein expression was induced with 0.5mM IPTG and the cells were grown at 37°C for an additional 3 hrs. Cells were harvested by centrifugation and resuspended in lysis buffer (20mM HEPES, pH 7.5, 150mM KCl, 0.01% Triton X-100, 10mM imidazole, 1mM PMSF and 10% glycerol). Cells were lysed by sonication, and the protein was purified using a Ni-NTA column, followed by ion-exchange chromatography using a Mono-Q column.

Electrophoretic mobility shift assay (EMSA)

a) EMSA experiment for RNA-PNA interaction: The RNA-PNA interaction analysis using EMSA was performed as per previously published protocols.[6-9] Briefly, the lyophilized samples of 26-mer RNA or PNA were dissolved in appropriate volume of RNA binding buffer (20mM HEPES, 150mM NaCl, 0.5mM EDTA, pH 7.0) to get the desired stock concentration. The RNA samples were heated to 95°C for 5 min for complete denaturation and then snap cooled on ice for 10 min. Each of the PNA oligomers (PNA1, PNA2 or PNA3) was added to a separate RNA sample, and the samples were incubated at room temperature (RT) for 30 min. Samples were analyzed by 12% native PAGE (acrylamide/bisacrylamide 19:1, w/w) in 1X TBE buffer (pH 8.3) for 1hr 15mins at 70V. Electrophoresis was performed at room temperature, and the gels were visualized using ethidium bromide staining. For dose-dependent EMSA experiments, varying concentrations of PNA1 (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10 and 20μM) or PNA2

(0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 10 µM) were used with 4µM 26-mer RNA. Full-length 4.5S RNA-PNA interaction was analysed similarly on a 7% native PAGE.

b) EMSA experiment for RNA-Ffh interaction: The RNA-Ffh interaction analysis using EMSA was performed as per previously published protocols.[10] EMSA experiments for visualizing the interaction of full length RNA (114-mer) with Ffh was performed on 7% native PAGE (acrylamide/bisacrylamide 19:1, w/w). RNA was incubated with Ffh in RNA binding buffer (20mM HEPES, 150mM NaCl, 0.5mM EDTA, pH 7.0) at 25°C for 15 min. Electrophoresis was performed in electrophoresis buffer (50 mM Tris acetate, 75 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 6.5) at 80V at 4°C. Buffer was exchanged every 30 min. Gels were visualized using ethidium bromide staining. For competition experiments with PNA, the heat denatured RNA was incubated with PNA molecules for 10 min, followed by incubation with Ffh for another 30 min, before loading on a 7% native PAGE as described above. For dose-dependent effect of PNA on Ffh-RNA interaction, varying concentrations of PNA1 or PNA2 (1.0, 2.5, 5.0, 7.5 and 10 µM) were used with 2µM 4.5S RNA and 4µM Ffh.

Circular dichroism (CD)

The 26-mer RNA was heated to 95°C for 5 min for complete denaturation and then snap cooled on ice for 10 min. Each of the PNA oligomers (PNA1, PNA2, PNA3 or PNA1-CPP) at 5 µM concentration was mixed in binding buffer (5mM Na_2HPO_4, 0.1mM EDTA, 100mM NaCl, pH 7.0) and incubated with RNA (1µM) at RT for 30mins. CD spectra were recorded in the range 200-320nm with 50nm/s scan rate, 1nm band width, 3 accumulations using a CD spectrophotometer (JASCO, J-815, Japan). Each plot is an average of at least 9 different scans. The data were plotted using Kaleidagraph software (Synergy, USA). The full-length RNA and PNA interaction study was performed under identical conditions.

GTPase assay

The GTPase assay was performed as published previously[11, 12] with some modifications. The 4.5S RNA (0.5µM) was heated to 95°C for 5 min for complete denaturation and then snap cooled on ice for 10 min. Each of the PNA oligomers (PNA1, PNA2, PNA3 or PNA1-CPP) was added (5 µM) to a separate RNA sample, and the samples were incubated at room temperature (RT) for 30 min. Wild-type Ffh (2µM), FtsY (47-497) (3µM), and GTP (1mM) were then added to these samples and incubated for 60 min at 37°C in buffer containing 50mM HEPES (pH 7.5), 150mM potassium acetate, 10mM magnesium acetate, 2mM DTT, 0.02 % Nikkol and 10% glycerol. The amount of GTP hydrolyzed was determined by a phosphate-binding assay using the dye malachite green.

Bacterial cell viability assay

E. coli AS19 cells, which are significantly more permeable to PNA than regular *E. coli* cells, were grown in Mueller-Hinton Broth (MHB) in the presence and absence of PNA1-CPP. A total of 200µl of the diluted bacterial suspension ($2.5x10^2$ CFU/mL) in MHB media was added into each well of a 96-well plate followed by indicated concentrations of PNA1-CPP. Water was used as a vehicle control. The plate was incubated at 37°C with orbital shaking in a multi-mode microplate reader (BioTek, USA). Each experiment contained three replicates, and the experiment was repeated thrice.

Spot assay

For the spot assay, AS19 cells were grown in MHB media with indicated concentrations of PNA1-CPP. A total of 200µl of the diluted bacterial suspension ($2.5x10^2$ CFU/mL) in MHB media was added into each 1.5mL tube. After 10 hr incubation, 10-fold serially diluted samples were spotted on Mueller-Hinton agar (MHA) plates. The plates were incubated at 37°C for 12 hrs and imaged using a gel doc system (G:Box Chemi-XRQ , Syngene, USA).

Bacterial growth recovery assay

For bacterial cell growth recovery assay, a plasmid (pSN1) coding for 4.5S DNA and another plasmid pET28a(+) coding for *lacI* repressor gene were co-transformed into AS19 cells (referred to here as AS19*). IPTG (1mM) was used as an inducer to overexpress the 4.5S RNA, and lac repressor (LacI) to control the leaky expression of 4.5S RNA. AS19* cells were grown with ampicillin (100µg/mL) and kanamycin (50µg/mL) as the selection markers. Recovery assay was performed with AS19* cells in the presence of 3µM PNA1-CPP in MHB medium supplemented

with appropriate antibiotics. For overexpression of 4.5S RNA in AS19* cells, 1mM IPTG was used. Cell-growth using the spot assay was performed as described above.

Transmission electron microscopy (TEM) experiment

E. coli AS19 or AS19* cells were grown in MHB media to an OD_{600} of 0.2. A total of 200µl of the diluted bacterial suspension ($2.5x10^2$ CFU/mL) in MHB media was added into separate 1.5mL tubes. Cells were incubated with PNA1-CPP at 37°C for 10 hrs and harvested by centrifugation. The cell pellets were washed with ultrapure water 4-5 times. Resuspended cells (5µL) were dispensed on a copper grid and allowed to dry inside a laminar hood. Subsequently, 5µL of 0.1% phosphotungstic acid was added and dried for 40 mins. Completely dried samples were subjected to TEM analysis using FEI Talos 200S system equipped with a 200kV Field Emission Gun (FEG).

References:

1. E. S. Andersen, M. A. Rosenblad, N. Larsen, J. C. Westergaard, J. Burks, I. K. Wower, J. Wower, J. Gorodkin, T. Samuelsson and C. Zwieb, *Nucleic Acids Res*, 2006, **34**, 163-168.
2. C. M. Courtney and A. Chatterjee, *ACS Infectious Diseases*, 2015, **1**, 253-263.
3. M. Kulik, A. Markowska-Zagrajek, M. Wojciechowska, R. Grzela, T. Witula and J. Trylska, *Biochimie*, 2017, **138**, 32-42.
4. M. Mondhe, A. Chessher, S. Goh, L. Good and J. E. Stach, *PLoS One*, 2014, **9(2)**: e89082.
5. P. Peluso, S.-O. Shan, S. Nock, D. Herschlag and P. Walter, *Biochemistry*, 2001, **40**, 15224-15233.
6. D.-F. K. Toh, G. Devi, K. M. Patil, Q. Qu, M. Maraswami, Y. Xiao, T. P. Loh, Y. Zhao and G. Chen, *Nucleic Acids Res.*, 2016, **44**, 9071-9082.
7. G. Devi, Z. Yuan, Y. Lu, Y. Zhao and G. Chen, *Nucleic Acids Res*, 2014, **42**, 4008-4018.
8. E. Peacey, L. Rodriguez, Y. Liu and M. S. Wolfe, *Nucleic Acids Res.*, 2012, **40**, 9836-9849.
9. T. Sato, Y. Sato and S. Nishizawa, *J. Am. Chem. Soc.*, 2016, **138**, 9397-9400.
10. G. Lentzen, B. Dobberstein and W. Wintermeyer, *FEBS Lett.*, 1994, **348**, 233-238.
11. T. Powers and P. Walter, *EMBO J.*, 1997, **16**, 4880-4886.
12. F. Y. Siu, R. J. Spanggord and J. A. Doudna, *RNA*, 2007, **13**, 240-250.