SUPPLEMENTARY MATERIALS

for Uemura et al. **"Crystal structure of the ligand binding form of nanoRNase from *Bacteroides fragilis*, a member of the DHH/DHHA1 phosphoesterase family of proteins"**

Supplementary Methods

S1. Protein preparation

The amplified fragment of the bfNrn gene (ORF ID: BF3670) was engineered into pET-21d and the resulting expression construct was used to transform *E. coli* Rosetta™2(DE3)pLysS cells (Merck, Darmstadt, Germany). Cells harboring the expression construct were cultured at 37°C in 1.5 l LB medium containing 50 μg /ml ampicillin to a density of 1×10^8 cells/ml before addition of isopropyl β-D-1-thiogalactopyranoside (50 μg/ml). The culture was then continued for a further 6 h before harvesting the cells by centrifugation. The cell pellet was stored at –20°C until required. The following procedures were carried out at 4°C except for size exclusion chromatography. Frozen cells (8 g) were thawed, suspended in 80 ml of 50 mM Tris-HCl (pH 8. 0), 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 5 mM imidazole, and disrupted by sonication on ice. The lysate was then clarified by centrifugation (38,000 *g* for 60 min). The resultant supernatant was loaded onto a His-Bind resin (Merck) column (bed volume, 10 ml) equilibrated with 20 mM Tris-HCl (pH 8. 0), 500 mM NaCl and 5 mM imidazole. Bound proteins were eluted with a linear gradient of 0.05–0.5 M imidazole (total volume, 200 ml). Fractions containing the target protein were pooled and concentrated with a Vivaspin (10,000 molecular weight cutoff) concentrator. The concentrated protein solution was then subjected to size exclusion chromatography using a Superdex 75 HR 10/30 column (GE Healthcare Biosciences) equilibrated with 20 mM Tris-HCl (pH 7.5), 100 mM NaCl. Chromatography was carried out on an ÄKTA explorer system (GE Healthcare Biosciences) at 20°C. Fractions containing the target protein (identified by SDS-PAGE) were concentrated and stored at 4°C.

The concentration of the purified protein was determined using the molar absorption coefficient at 280 nm calculated with the Protpram program of Expert Protein Analysis System (ExPASy) proteomics server (http://www.expasy.org/). Approximately 3.5 mg bfNrn was obtained from 8 g of cells.

S2. Exonuclease activity assay

The assay was performed as described previously [1]. Briefly, the reaction mixture (10 µl) contained 50 mM HEPES-KOH (pH 7.5), 100 mM KCl, 5 mM $MnCl_2$ (or 5 mM EDTA), 10 nM 5'-^{32}P-labeled 11-mer ssDNA and 1 µM bfNrn. The assay was carried out using various substrates of different length i.e., 10 nM 5'-^{32}P-labeled ssDNA (3, 6, or 11-mer), 10 µM cold ssDNA (3, 6, or 11-mer) and 500 nM bfNrn in 50 mM HEPES-KOH, pH 7.5, 100 mM KCl and 5 mM $MnCl_2$. The mixture was incubated at 37°C before stopping the reaction by addition of 1 µl of 100 mM EDTA and 11 µl of phenol/chloroform. Analysis of the degradation product by mass spectrometry was performed as described previously [2].

S3. Phosphatase activity assay

The reaction mixture (100 µl) contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MnCl_2$, 25 µM adenosine 3',5'-bisphosphate (pAp), guanosine 3',5'-bisphosphate (pGp), adenosine 2',5'-bisphosphate, 3'-AMP, or c-di-GMP and 500 nM bfNrn. Reactions were incubated at 37°C for 6 h and then stopped by addition of 20 µl of 100 mM EDTA. An equal volume (120 µl) of buffer I (50 mM Tris-HCl (pH 7.5), 5 mM tetra-*n*-butylammonium hydroxide and 10% methanol) was added, and the protein was removed by ultrafiltration using a membrane filter (10,000 molecular weight cutoff). A 200-µl aliquot of the filtrate was applied to a reversed-phase column (CAPCELL PAK C18 (Shiseido)) equilibrated with buffer I. Bound material was subsequently eluted using a gradient of 10 to 50% methanol. To compare the phosphatase activity, the inorganic phosphate was measured using a

colorimetric assay [3].

Supplementary Tables

Supplementary Table S1. Data collection and refinement statistics.

Dataset	bfNrn-Mn^{2+}-GMP complex	
Wavelength (Å)	1.000	1.892
Resolution (Å)	50–2.95 (3.00-2.95)	50–3.20 (3.26-3.20)
Space group	$P3_221$	$P3_221$
Cell constants (Å) a, b, c	90.8, 90.8, 107.9	90.9, 90.9, 108.0
Cell angles (°) α, β, γ	90, 90, 120	90, 90, 120
Data collection		
Observed reflections	205804	91960
Unique reflections	11209 (556)	8823 (438)
Completeness (%)	99.8 (100)	99.5 (100)
Redundancy	21.9 (22.3)	10.4 (10.9)
$I/\sigma(I)$	48.6 (12.8)	42.3 (10.4)
R_{merge} (%)[a]	9.2 (30.2)	10.3 (29.9)
Refinement[d]		
R-factor (%)[b]	22.09	
R_{free} (%)[c]	26.08	
Average B-factor ($Å^2$)	61.2	
Stereochemistry r.m.s d[e]		
Bonds lengths (Å)	0.007	
Bond angles (°)	1.313	

[a] Values in parentheses are for the outermost shell.

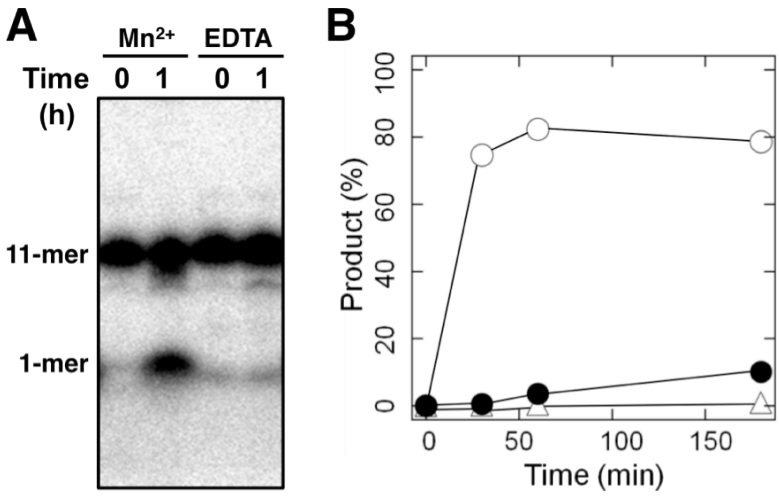
[b] $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl)-I(hkl)|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $I(hkl)$ is the averaged intensity for multiple measurements.

[c] *R*-factor $= \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$

[d] R_{free} is monitored with 5% of the reflection data excluded from refinement.

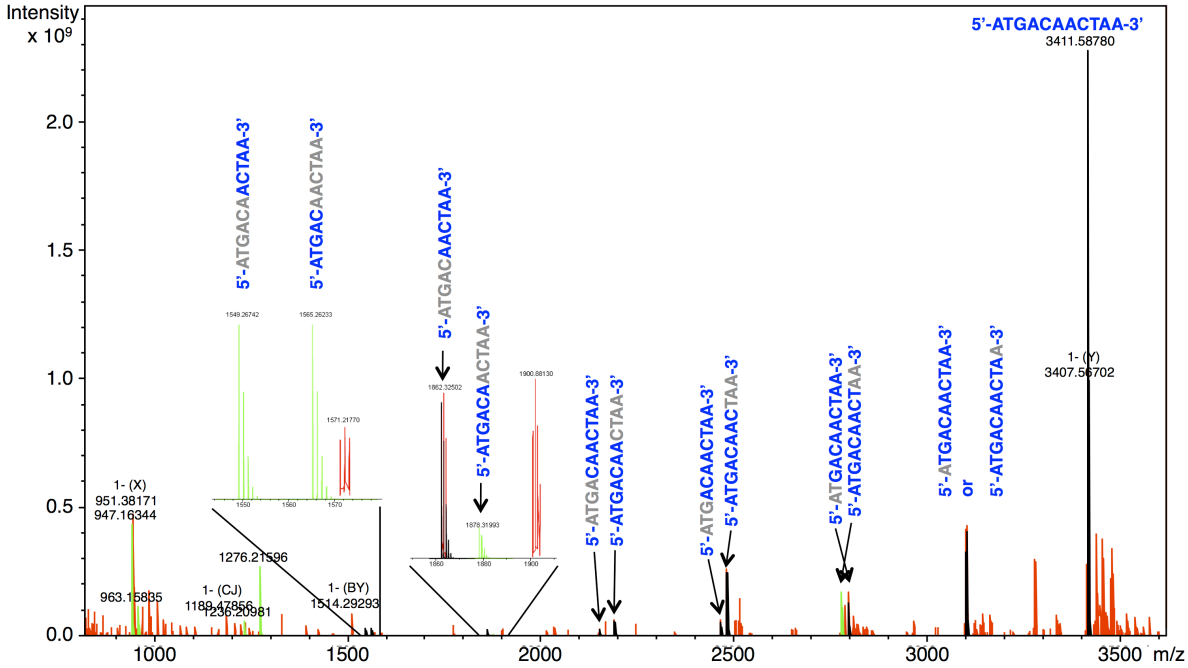
[e] Over 90% of main-chain dihedrals fall within the 'most favored regions' of the Ramachandran plot.

Supplementary Figures



Supplementary Fig. S1. Exonuclease activity of bfNrn. (A) Dependence of enzyme activity on Mn^{2+}. An aliquot of 10 nM 5'-^{32}P-labeled 11-mer oligodeoxyribonucleotide was reacted with 1 μM bfNrn at 25°C. The reaction mixtures contained 50 mM HEPES (pH 7.5), 100 mM KCl and 5 mM $MnCl_2$ or 50 mM EDTA. (B) Dependence on substrate length. Reactions were performed as described in (A) except for a lower concentration of bfNrn (0.5 μM) and the addition of 10 μM unlabeled oligodeoxyribonucleotide. Circles, 3-mer; filled circles, 6-mer; triangles, 11-mer.

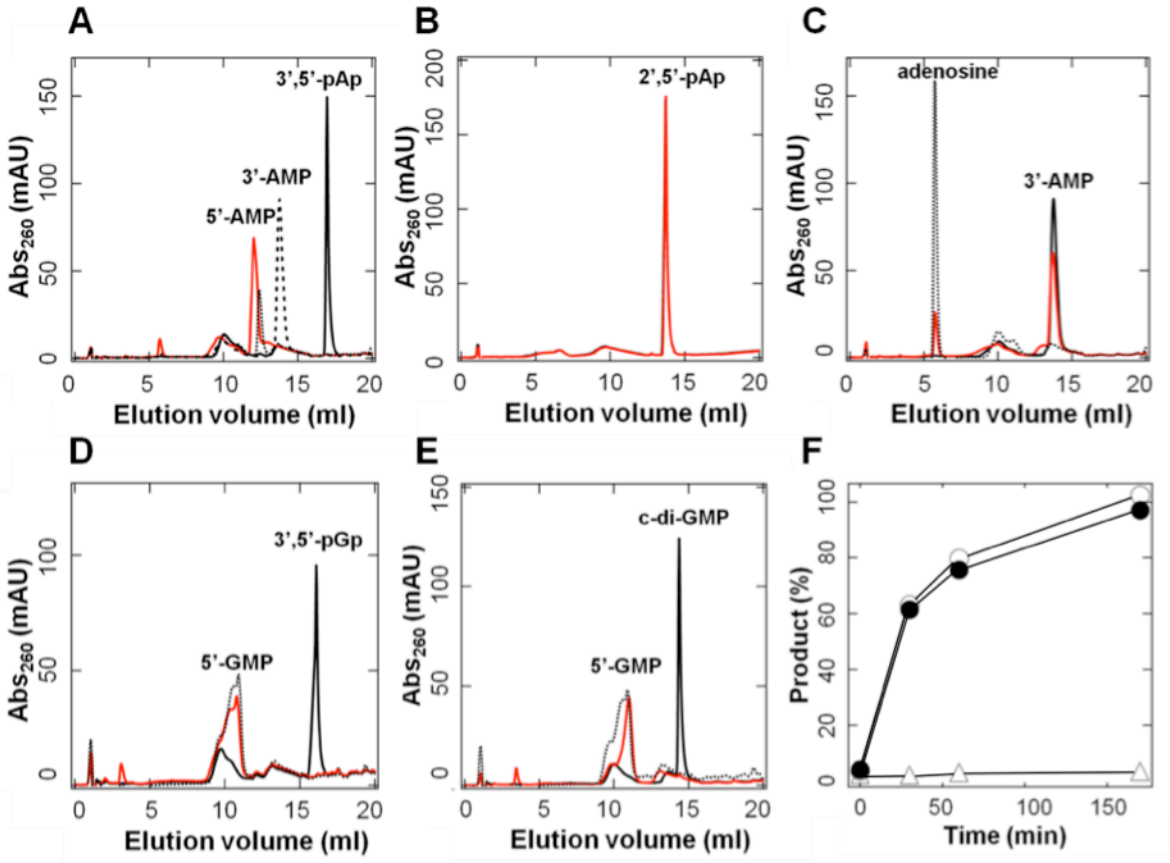
A



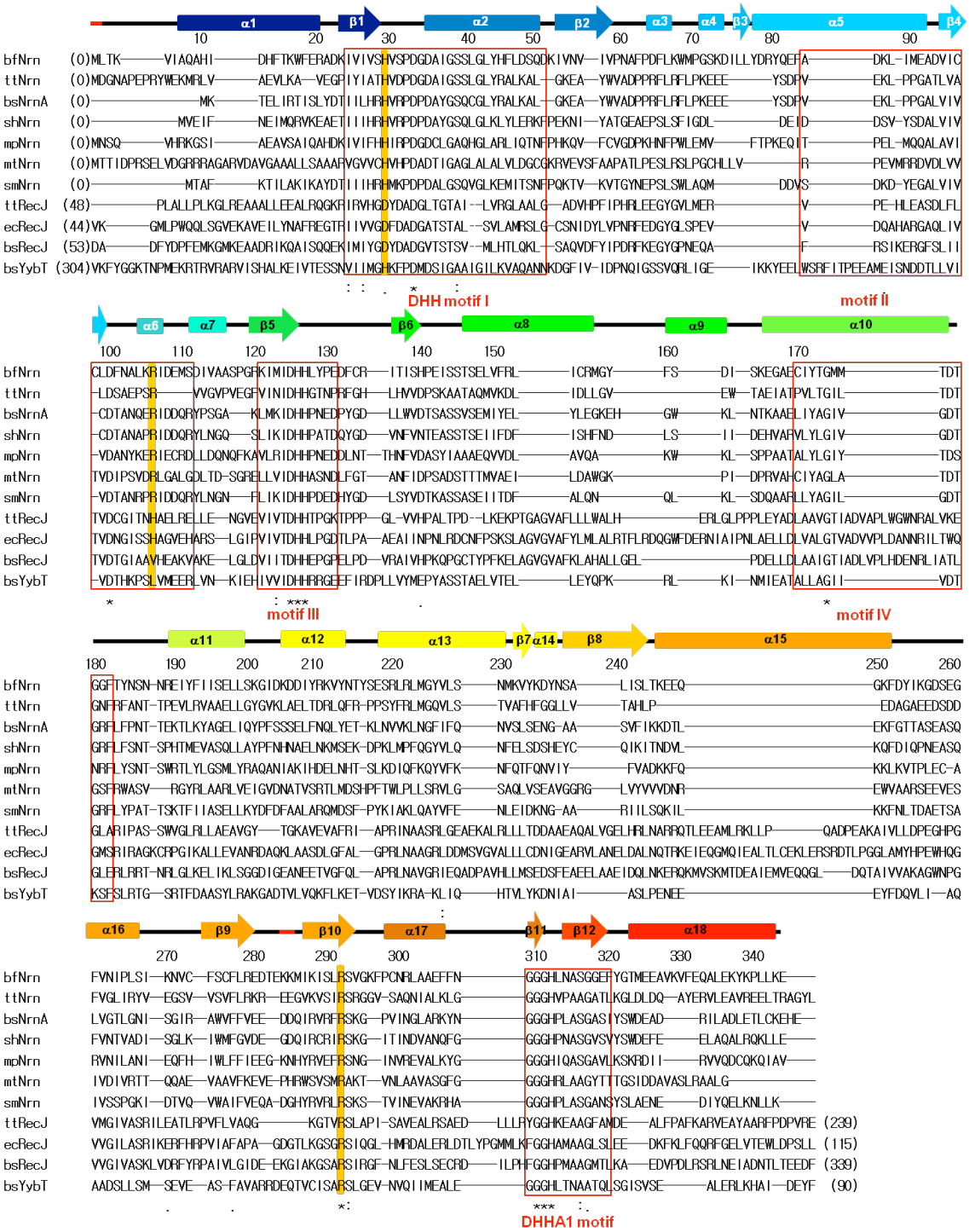
B

11-mer	substrate	5'-ATG-ACA-ACT-AA-3'	C 108 H136 N45 O 63 P 11	3410.59
10-mer	(5' end digested)	5'-TG-ACA-ACT-AA-3'	C 98 H124 N 49 O 58 P 10	3097.53
10-mer	(3' end digested)	5'-ATG-ACA-ACT-A-3'	C 98 H124 N 49 O 58 P 10	3097.53
9-mer	(5' end digested)	5'-G-ACA-ACT-A-3'	C 88 H113 N 32 O 55 P 9	2793.48
9-mer	(3' end digested)	5'-TG-ACA-ACT-3'	C 88 H113 N 32 O 45 P 9	2775.46
8-mer	(5' end digested)	5'-CA-ACT-AA-3'	C 78 H99 N 33 O 55 P 8	2464.43
8-mer	(3' end digested)	5'-ATG-ACA-A-3'	C 78 H99 N 33 O 55 P 8	2480.43
7-mer	(5' end digested)	5'-CA-ACT-AA-3'	C 68 H87 N 28 O 40 P 7	2151.37
7-mer	(3' end digested)	5'-ATG-ACA-A-3'	C 69 H 87 N 30 O 40 P 7	2191.38
6-mer	(5' end digested)	5'-A-ACT-AA-3'	C 59 H 75 N 25 O 34 P 6	1862.33
6-mer	(3' end digested)	5'-ATG-ACA-3'	C 59 H 75 N 25 O 35 P 6	1878.32
5-mer	(5' end digested)	5'-ACT-AA-3'	C 49 H 63 N 20 O 29 P 5	1549.27
5-mer	(3' end digested)	5'-ATG-AC-3'	C 49 H 63 N 20 O 30 P 5	1565.26
4-mer	(5' end digested)	5'-CT-AA-3'	C 39 H 51 N 15 O 24 P 4	1236.21
4-mer	(3' end digested)	5'-ATG-A-3'	C 40 H 51 N 17 O 24 P 4	1276.22
3-mer	(5' end digested)	5'-T-AA-3'	C 30 H 39 N 12 O 18 P 3	947.16
3-mer	(3' end digested)	5'-ATG--3'	C 30 H 39 N 12 O 19 P 3	963.16
2-mer	(5' end digested)	5'-AA-3'	C 20 H 26 N 10 O 11 P 2	643.12
2-mer	(3' end digested)	5'-AT-3'	C 20 H 27 N 7 O 13 P 2	634.11
1-mer		5'-A	C 10 H 14 N 5 O 6 P 1	330.06
		5'-T	C 10 H 15 N 2 O 8 P 1	321.05
		5'-G	C 10 H 14 N 5 O 7 P 1	346.06
		5'-C	C 9 H 14 N 3 O 7 P 1	306.05

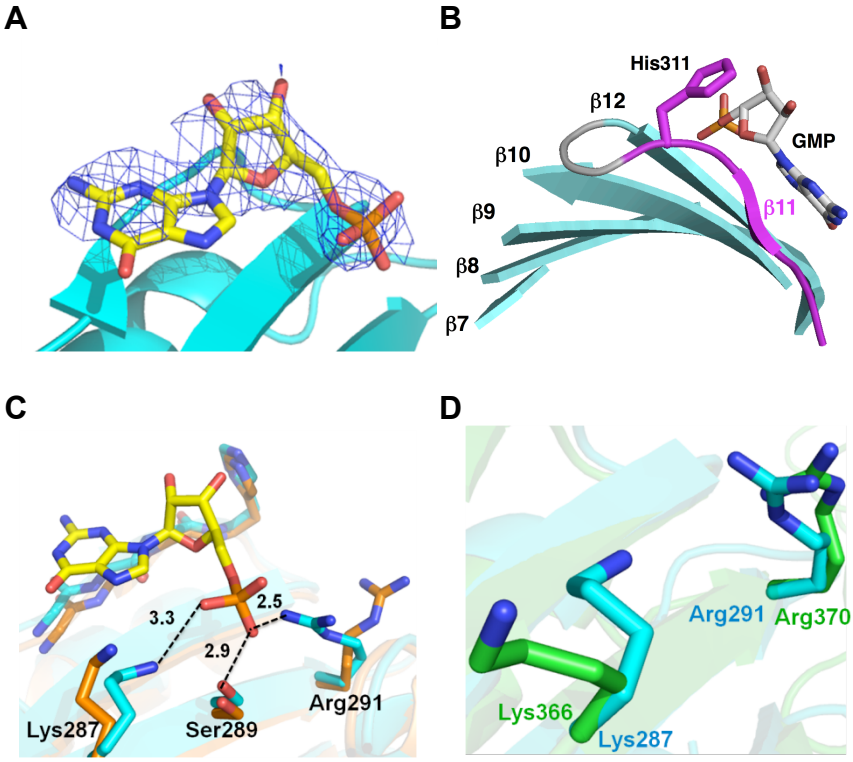
Supplementary Fig. S2. Mass spectrometric analysis of reaction products by bfNrn. Reactions were performed as described in Fig. S1A except for a longer reaction time (11 h). Other procedures were perfomed as described [2]. (A) The deconvoluted spectrum of reacted products of 5'-phosphorylated 11-mer ssDNA. For clarity, information concerning the major species are shown above the corresponding peaks. (B) Theoretical masses of each DNA fragment. These were calculated assuming that bfNrn hydrolyzes a phosphodiester bond at 5'-side or 3'-side of a phosphate.



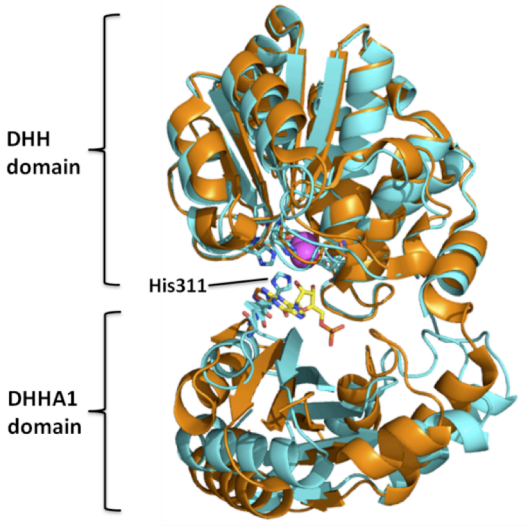
Supplementary Fig. S3. Phosphatase activity of bfNrn. (A–D) HPLC profile of the products of bfNrn phosphatase activity on 3',5'-pAp (A), 2',5'-pAp (B), 3'-AMP (C), 3',5'-pGp (D) and c-di-GMP (E) substrate. Elution profiles of the product of hydrolysis by bfNrn (red lines) and respective control mixtures (black lines) are shown. An aliquot of 25 μM substrate was reacted with 0.5 μM bfNrn at 37°C for 6 h. The reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl and 5 mM $MnCl_2$. (F) Time course of reactions mediated by bfNrn. Circles, pAp; filled circles, pGp; triangles, 3'-AMP.



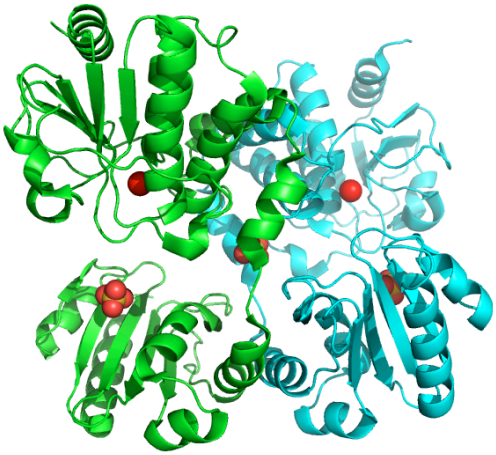
Supplementary Fig. S4. Sequence alignments of bfNrn, its homologs and other DHH/DHHA1 family proteins. The secondary structure of bfNrn is shown above the sequences; the horizontal bars indicate α-helices and arrows indicate β-strands. The five motifs proposed by Aravind and Koonin [4] are highlighted in red boxes. Residues thought to be involved in binding to a ligand are highlighted by an orange background. Species name are as follows: bf, *Bacteroides fragilis*; tt, *Thermus thermophilus*; bs, *Bacillus subtilis*; sh, *Staphylococcus haemolyticus*; mp, *Mycoplasma pneumoniae*; mt, *Mycobacterium tuberculosis*; sm, *Streptococcus mutans*; and ec, *Escherichia coli.* The sequence alignment was prepared using Clustal omega [5].



Supplementary Fig. S5. GMP-binding site. (A) The Fo-Fc electron density map (blue mesh) for the GMP is contoured at the 1.0 σ level. (B) Conformation of β11 strand formed by the DHH1A motif. (C) Comparison between Mn^{2+}-GMP complex (cyan sticks) and ligand-free form of bfNrn (orange sticks; PDB ID: 3DMA). (D) Superimposition of Lys287 and Arg291 of bfNrn (cyan sticks) with the corresponding residues of ttRecJ (green sticks; PDB ID: 2ZXP). The dashed lines indicate the distances (Å).



Supplementary Fig. S6. Putative domain closure of bfNrn to adopt a catalytically active conformation. A model structure after domain closure (cyan) was constructed by moving the DHHA1 domain to the DHH domain using Pymol. The ligand-free form of bfNrn (orange, PDB ID: 3DMA) is superimposed.



Supplementary Fig. S7. Position of sulfate ions in the structure of shNrn dimer (PDB ID: 3DEV). Each subunit is drawn in a different color. The position of each Mg^{2+} ion is shown as a red sphere. Three sulfate ions are shown as space-filling models colored by element type.

Supplementary References

[1] Wakamatsu, T., Kim, K., Uemura, Y., Nakagawa, N., Kuramitsu, S. and Masui, R. (2011) Role of RecJ-like protein with 5'-3' exonuclease activity in oligo(deoxy)nucleotide degradation. J. Biol. Chem. 286, 2807-2816.

[2] Fukui, K., Takahata, Y., Nakagawa, N., Kuramitsu, S. and Masui, R. (2007) Analysis of a nuclease activity of catalytic domain of *Thermus thermophilus* MutS2 by high-accuracy mass spectrometry. Nucleic Acids Res. 35, e100.

[3] Wakamatsu, T., Nakagawa, N., Kuramitsu, S. and Masui, R. (2008) Structural basis for different substrate specificities of two ADP-ribose pyrophosphatases from *Thermus thermophilus* HB8. J. Bacteriol. 190, 1108-1117.

[4] Aravind, L. and Koonin, E.V. (1998) A novel family of predicted phosphoesterases includes Drosophila prune protein and bacterial RecJ exonuclease. Trends Biochem. Sci. 23, 17-19.

[5] Sievers, F. et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.