Supporting Information

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SI Text

Materials and General Methods. All chemical reagents were purchased from Sigma-Aldrich, unless otherwise indicated. Primers were purchased from Integrated DNA Technologies. UV-vis absorption spectroscopy was performed on an Agilent 8453 Diode Array spectrophotometer. Anaerobic procedures were carried out in a custom-designed MBraun glovebox equipped with a chiller at 15 °C. All solutions and proteins were made anaerobic on a Schlenk line by three cycles of evacuation (5 min) followed by flushing with Ar gas (10 min) before being brought into the glovebox. Nucleotides, *S*-adenosylmethionine (SAM), and NADPH were brought into the glovebox as lyophilized solids. *Escherichia coli* thioredoxin (TrxA) and thioredoxin reductase (TrxB) were purified according to published procedures (1, 2).

Cloning, Expression, and Purification of the NrdD and NrdG Proteins from *N. bacilliformis* and NrdD from the Deep-Branching Thermophilic Bacterium *T. maritima*. The genes were amplified from the respective genomic DNA by PCR using Phusion polymerase (NEB) and the primers TmNrdDf/r, NbNrdDf/r, and NbNrdGf/r (Table S2) and were inserted into pET28a (Novagen), and linearized with NdeI and HindIII by Gibson isothermal assembly (3), yielding the plasmids pET28a-NrdD protein from *N. bacilliformis* (NbNrdD), pET28a-NrdG protein from *N. bacilliformis* (NbNrdG), and pET28a-NrdD from the deep-branching thermophilic bacterium *T. maritima* (TmNrdD). Because of the insolubility of the protein expressed from pET28a-NbNrdG, the *nrdG* gene was excised using the restriction enzymes NdeI and XhoI and ligated into pSV272, which contains a maltose-binding protein (MBP) with an N-terminal His_6-tag, and linearized with the same enzymes to obtain pMBP-NbNrdG. The plasmids pET28a-NbNrdD(C300A), pET28a-NbNrdD(C301A), and pET28a-NbNrdD(E438Q), containing the C300A, C301A, and E438Q mutations, respectively, were constructed by site-directed mutagenesis from pET28a-NbNrdD using the primers NbNrdD(C300A)f/r, NbNrdD(C301A)f/r, and NbNrdD(E438Q)f/r (Table S2). All constructs were confirmed by DNA sequencing at the Massachusetts Institute of Technology Biopolymers Laboratory. The pET28a plasmid contains an N-terminal His_6 affinity purification tag followed by a thrombin cleavage site (MGSSH HHHHH SSGLV PRGSH-).

Expression and purification of the three proteins followed a similar protocol. The plasmids pET28a-TmNrdD, pET28a-NbNrdD, and pMBP-NbNrdG were separately transformed into BL21 (DE3) codon plus (RIL) cells (Stratagene) and grown on Luria-Bertani (LB)-agar plates with 50 μg/mL kanamycin (Kan). A single colony was inoculated into 5 mL starter culture of LB (50 μg/mL Kan in all growths), grown at 37 °C until saturated (12 h), and transferred into 200 mL of LB. Media for expression of NbNrdD and TmNrdD were supplemented with 50 μM $ZnSO_4$. The cultures were grown at 37 °C with shaking at 200 rpm. At OD_{600} ~ 0.6, the temperature was decreased to 20 °C, and isopropyl β-D-1-thiogalactopyranoside (Promega) was added to a final concentration of 0.1 mM. After 12 h, cells were pelleted by centrifugation (4,000 × *g*, 10 min, 4 °C) and frozen at −80 °C. Typical yield was ~5 g of cell paste/L.

Cell paste (~1 g) was resuspended in 25 mL of 20 mM Tris, pH 7.5, 5 mM DTT, and 1 mM PMSF (buffer A). The cells were lysed by a single passage through a French pressure cell (14,000 psi). DNA was precipitated by dropwise addition of 5 mL of buffer A containing 6% (wt/vol) streptomycin sulfate. The mixture was shaken for an additional 10 min, and the precipitated DNA was removed by centrifugation (20,000 × *g*, 10 min, 4 °C). Solid $(NH_4)_2SO_4$ was then added to 60% (wt/vol) saturation. The solution was shaken for an additional 20 min, and the precipitated protein was isolated by centrifugation (20,000 × *g*, 10 min, 4 °C).

The pellet was dissolved in 30 mL of 20 mM Tris, pH 7.5, 5 mM Tris(2-carboxyethyl)phosphine (TCEP), and 0.2 M KCl (buffer B) and incubated with 2 mL of TALON resin (Clontech) with shaking for 30 min. The column was then packed (0.8 × 4 cm) and washed with 10 column volumes of buffer B, followed by 10 column volumes of buffer B containing 5 mM imidazole. Protein was eluted with 5 column volumes of buffer B containing 150 mM imidazole. The eluted protein was precipitated with solid $(NH_4)_2SO_4$ to 60% saturation and isolated by centrifugation (20,000 × *g*, 10 min, 4 °C). The pellet was dissolved in 0.5 mL of buffer B and desalted using a Sephadex G-25 column (1.5 × 8.5 cm, 15 mL), preequilibrated with 20 mM Tris, pH 7.5, 5% (vol/vol) glycerol, and 1 mM DTT (buffer C). The eluted protein was concentrated to ~200 μM by ultrafiltration (Amicon YM-30), frozen in aliquots in liquid N_2, and stored at −80 °C. The final yield was ~10 mg/g cells for NbNrdD (ε_{280} = 73,410 $M^{-1}\cdot cm^{-1}$) and ~1 mg/g cells for MBP-NbNrdG (ε_{280} = 95,760 $M^{-1}\cdot cm^{-1}$). SDS/PAGE gels of the purified proteins are shown in Fig. S7.

Preparation of [SeMet]-Labeled TmNrdD for Crystallography. The procedure was adapted from existing protocols (4). A 5 mL starter culture was prepared according to the procedure described above, harvested by centrifugation (3,000 × *g*, 10 min, 4 °C), and transferred into 200 mL of M9 minimal medium supplemented with glucose (0.4%), thiamine (50 mg/L), Kan (50 mg/L), $ZnSO_4$ (50 μM), $FeCl_3$ (10 μM), $MgCl_2$ (2 mM), $CaCl_2$ (0.1 mM), and the L-amino acids lysine (100 mg/L), phenylalanine (100 mg/L), threonine (100 mg/L), isoleucine (50 mg/L), leucine (50 mg/L), and valine (50 mg/L). The culture was grown at 37 °C with shaking at 220 rpm. At OD_{600} ~ 0.3, SeMet (50 mg/L) was added, followed by shaking for 20 min. The temperature was lowered to 20 °C, and IPTG (Promega) was added to a final concentration of 0.2 mM. After 12 h, cells were pelleted by centrifugation (4,000 × *g*, 10 min, 4 °C) and frozen at −80 °C. The yield was ~1 g of cell paste, and purification was carried out according to the procedure described for NbNrdD. The final yield was ~2 mg/g cells (ε_{280} = 106,830 $M^{-1}\cdot cm^{-1}$). An SDS/PAGE gel of the purified protein is shown in Fig. S7.

1. Russel M, Model P (1985) Direct cloning of the *trxB* gene that encodes thioredoxin reductase. *J Bacteriol* 163(1):238–242.
2. Chivers PT, et al. (1997) Microscopic pKa values of *Escherichia coli* thioredoxin. *Biochemistry* 36(48):14985–14991.
3. Gibson DG, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343–345.
4. Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J (1993) Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J Mol Biol* 229(1):105–124.

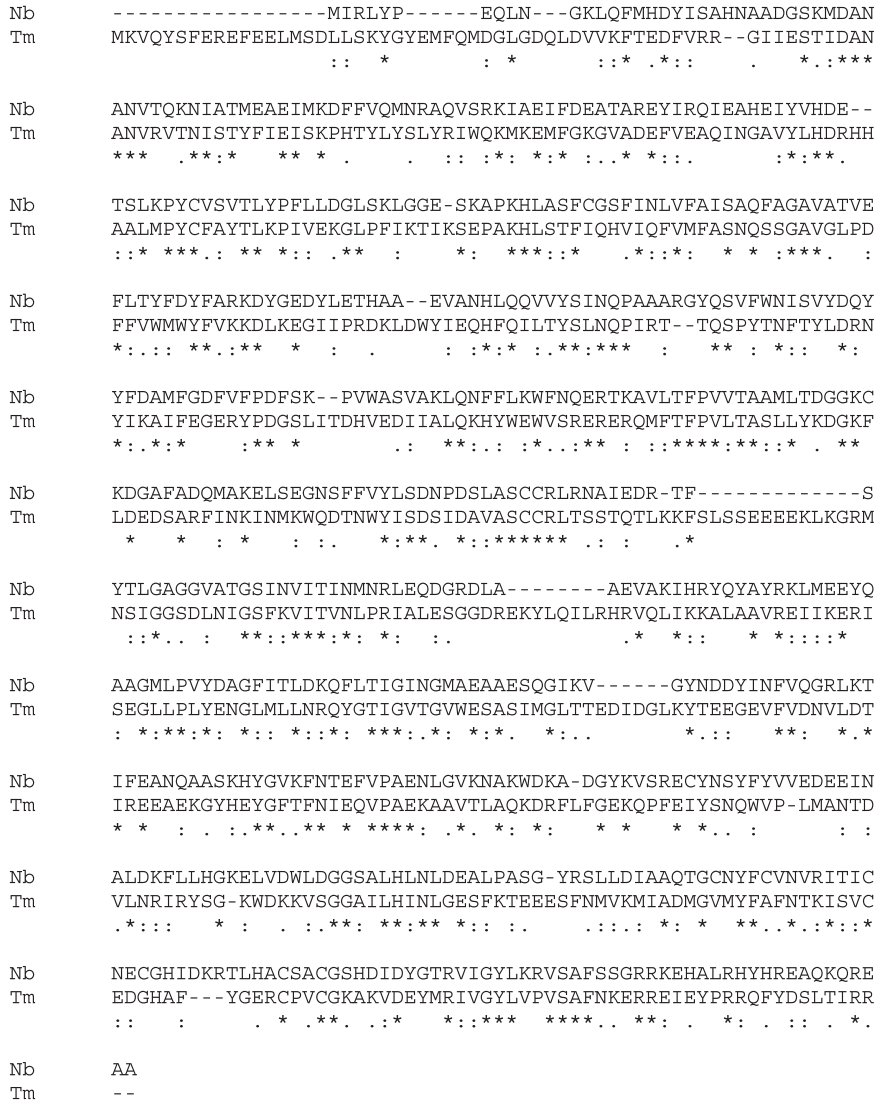

Fig. S1. Sequence alignment of *N. bacilliformis* (Nb) and *T. maritima* (Tm) NrdD created using Clustal Omega (1).

1. Sievers F, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7(1):539.

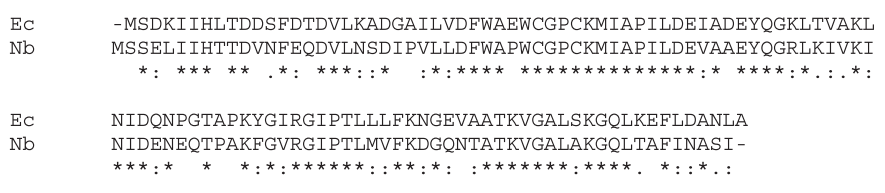

Fig. S2. Sequence alignment of *E. coli* (Ec) and *N. bacilliformis* (Nb) TrxA created using Clustal Omega (1).

1. Sievers F, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7(1):539.

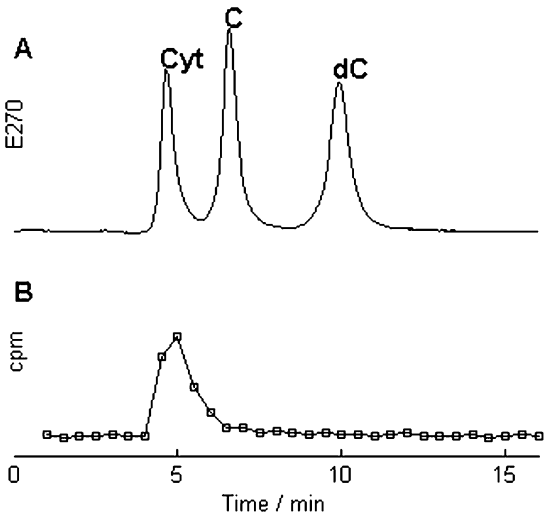

Fig. S3. HPLC detection of Cyt released in the reaction of NbNrdD(C300A) with 5-[^{3}H]-CTP. (*A*) Standards monitored by absorbance at 270 nm. (*B*) 5-[^{3}H]-Cyt detected by scintillation counting.

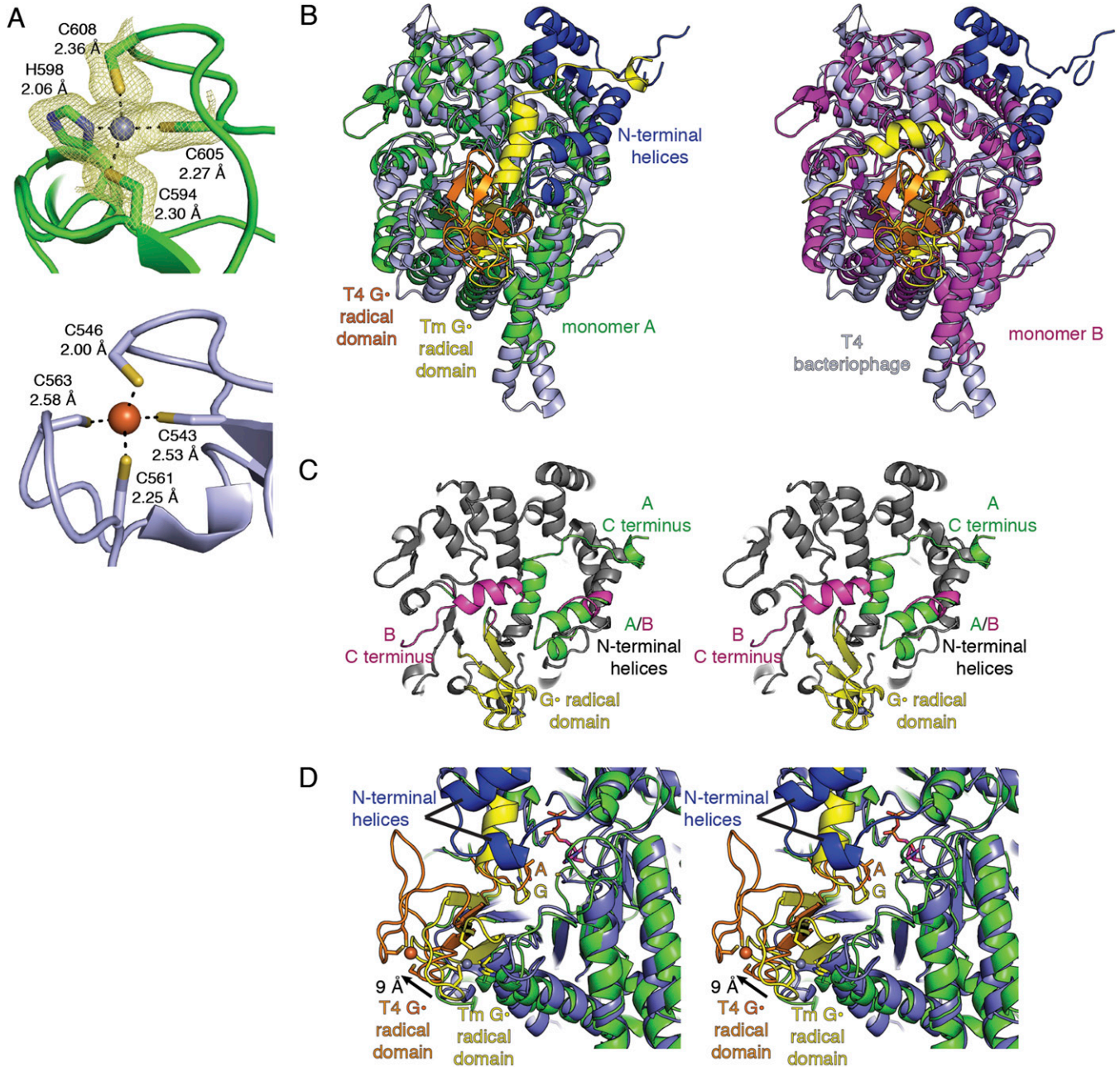

Fig. S4. Comparison of *T. maritima* and T4 bacteriophage class III ribonucleotide reductases (RNRs). (*A*) The Zn-binding site of TmNrdD (green, monomer A) is similar to the T4 bacteriophage NrdD (light blue) [Protein Data Bank (PDB) ID code 1H79] (1) with the exception that H598 replaces C563. Composite omit maps around the Zn and ligating residues are contoured at 1σ (yellow). (*B*) Comparison of TmNrdD monomer A (green) and TmNrdD monomer B (magenta) to the T4 bacteriophage NrdD (light blue). The N-terminal helices in TmNrdD (dark blue) have no counterpart in the T4 NrdD. The T4 G• domain (orange) and Tm G• domain (yellow) are shifted as seen in Fig. 5*D*. (*C*) Stereoimage of Fig. 5*C*. Monomers of TmNrdD differ by more than 1 Å in two regions: the tip of the N-terminal helices that contact the G• domain and the C-terminal helix (green, monomer A; magenta, monomer B). The core of the protein is unchanged (gray). (*D*) Stereoimage of Fig. 5*D*. G• domains of TmNrdD (yellow, Gly shown in sticks) in both chains are positioned further inside the barrel relative to that of T4 bacteriophage (orange, G580A in sticks). The C-terminal helix of TmNrdD is shown in its monomer A orientation (yellow). Zinc (TmNrdD, gray) and iron (T4 phage, orange) atoms, from Tm and T4 structures, respectively, are shown as spheres. CDP (magenta) is modeled based on the *T. maritima* class II RNR structure (PDB ID code 1XJN) (2).

1. Larsson K-M, Andersson J, Sjöberg B-M, Nordlund P, Logan DT (2001) Structural basis for allosteric substrate specificity regulation in anaerobic ribonucleotide reductases. *Structure* 9(8): 739–750.
2. Larsson K-M, et al. (2004) Structural mechanism of allosteric substrate specificity regulation in a ribonucleotide reductase. *Nat Struct Mol Biol* 11(11):1142–1149.

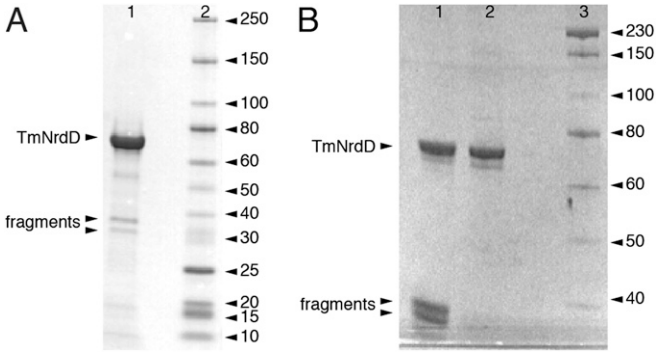

Fig. S5. TmNrdD fragments form during purification and are enriched by crystallization (SDS/PAGE, 10% gels). (*A*) Purified TmNrdD contains intact enzyme (75 kDa) and a small amount of two fragments (~36 and ~39 kDa). Molecular weight markers in lane 2. (*B*) Unwashed crystals of TmNrdD (lane 1) show enrichment of the fragment bands, whereas the surrounding protein-precipitant solution (lane 2) is depleted in the fragmented protein. This differential suggests selective crystallization of the two fragments over intact enzyme. Molecular weight markers in lane 3.

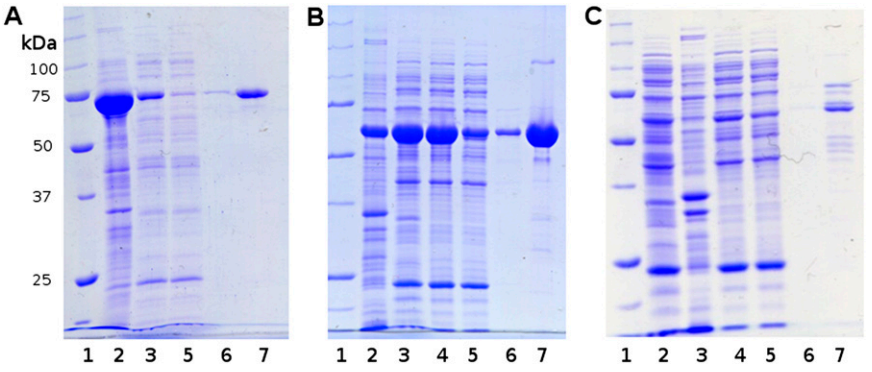

Fig. S6. Purification of (*A*) [SeMet]-TmNrdD; (*B*) NbNrdD, and (*C*) MBP-NbNrdG (SDS/PAGE 10% gels). Lanes: (1) ladder; (2) streptomycin sulfate precipitate; (3) streptomycin sulfate supernatant; (4) ammonium sulfate precipitate; (5) TALON column flow-through; (6) TALON column wash; and (7) TALON column eluted protein.

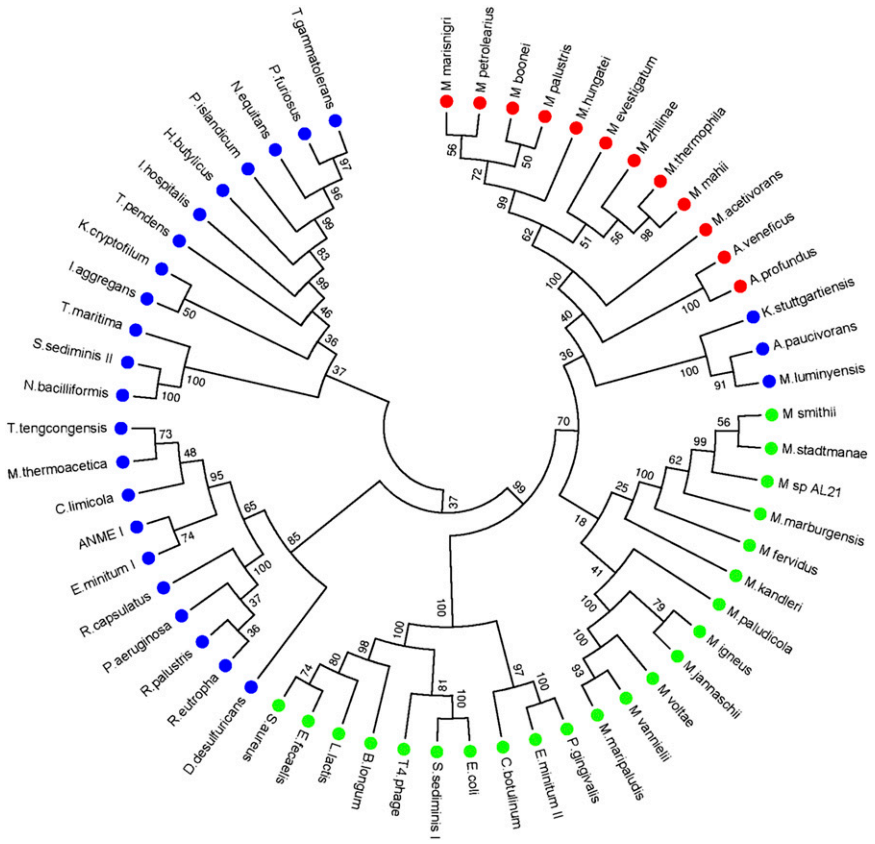

Fig. S7. Unrooted phylogenetic tree of NrdDs as shown in Fig. 7*B*. Bootstrap values resulting from 2,000 resamplings are given for each branch. Nrd1, Nrd2, and Nrd3 are labeled green, blue, and red, respectively.

Table S1. Data collection and refinement statistics

	SeMet TmNrdD
Data collection and processing*	
Dataset	Se peak
Resolution (Å)	50–1.64 (1.67–1.64)
Completeness %	94.4 (65.4)
<I/σI>	6.6 (1.1)[†]
R_{sym}	0.059 (0.64)
Unique reflections	279,509 (9,748)
Redundancy	1.8 (1.6)
CC1/2	(0.55)
Phasing	
Phasing resolution (Å)	50–1.80
Figure of merit (FOM)	0.42
Density modification FOM	0.67
Density modification R factor	0.25
Model refinement	
Resolution (Å)	50–1.64
R_{work}, R_{free}[‡]	0.171, 0.200
Protein atoms	10,331
Water molecules	892
RMS bond length deviation (Å)	0.050
RMS Bond angle deviation (°)	0.928
Rotamer outliers (%)	0.90
Average B factors (Å^2)	
Monomer A	21.6
Monomer B	20.6
Water	35.5

RMS, root mean square.

*Statistics were calculated with Friedel pairs kept separate during scaling. Numbers in parentheses are for the highest-resolution bin.

[†]Data were trimmed based on CC1/2 value rather than the <I/σI> value. Inclusion of highest resolution data improved the quality of the electron density maps.

[‡]A total of 3783 reflections were set aside before refinement for cross-validation.

Table S2. Primers used for cloning and site-directed mutagenesis

Name	Sequence
TmNrdDf	GCGGCCTGGTGCCGCGCGGCAGCCATATGAAGGTTCAGTATTCGTTCGAAAGAG
TmNrdDr	GGTGGTGCTCGAGTGCGGCCGCTCACCTTCTGATTGTCAGCG
NbNrdDf	GCGGCCTGGTGCCGCGCGGCAGCCATATGATTCGGCTGTATCCCGAACAG
NbNrdDr	GGTGGTGCTCGAGTGCGGCCGCCTAAGCCGCCTCGCGCTGCTTTTGC
NbNrdGf	GCGGCCTGGTGCCGCGCGGCAGCCATATGCCGAGCCTGAAATTCACCACCG
NbNrdGr	GGTGGTGCTCGAGTGCGGCCGCTCAGGCCGTCTGAACAAACGCCC
NbNrdD(C300A)f	CTCGCTCGCCTCCGCCTGCCGCCTGCGC
NbNrdD(C300A)r	GCGCAGGCGGCAGGCGGAGGCGAGCGAG
NbNrdD(C301A)f	CGCCTCCTGCGCCCGCCTGCGCAACGCC
NbNrdD(C301A)r	GGCGTTGCGCAGGCGGGCGCAGGAGGCG
NbNrdD(E438Q)f	CTCGCTCGCCTCCTCCTGCCGCCTGCGC
NbNrdD(E438Q)r	GCGCAGGCGGCAGGAGGAGGCGAGCGAG

Mutations are underlined.