Supporting Information

The HD-[HD-GYP] Phosphodiesterases; Activities and Evolutionary Diversification within the HD-GYP family

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Plasmid construction, cloning and mutagenesis of SO3491. The gene encoding for the HD-GYP protein from *Shewanella (S.) oneidensis* MR-1, SO3491 (NCBI accession: NP_719040), was inserted into the pSUMO expression vector (LifeSensors, Inc) to express the protein of interest as a linear chimera with the small ubiquitin-like modifier (SUMO) protein. The vector was kindly provided by Dr. Squire J. Booker (The Pennsylvania State University). This vector encodes for an N-terminal His_6-tag, the SUMO protein, and a Ulp1 recognition site for the subsequent cleavage of the appended SUMO tag. The wild-type (WT) and variant encoding genes of SO3491 were inserted in the PDB.His.MBP expression vector (Berkeley Structural Genomics Center) using the primers listed in Table S1 via the HindIII and XhoI restriction sites to express SO3491 as a fusion with the maltose binding protein (MBP). This vector encodes for an N-terminal His_6-tag, the MBP tag, and a Tobacco Etch Virus (TEV) recognition site prior to SO3491, to allow for the subsequent cleavage of MBP. Single-point amino acid substitutions using the primers listed in Table S1 were generated by back-to-back PCR (Q5 Mutagenesis, New England Biolabs, Ipswitch, MA).

Plasmid construction, cloning and mutagenesis of MBP-VCA0681. The plasmids expressing the WT and single-point variants of MBP-VCA0681 from *V. cholerae* were kindly provided by Dr. Donald M. Kurtz (Department of Chemistry, University of Texas at San Antonio).[1]

Protein expression and purification. All plasmids were transformed in T7 express *Escherichia (E.) coli* competent cells (NEB, Ipswitch, MA). Transformed cells were grown in minimal (M9) media to direct specific metal incorporation in the presence of 0.5% (w/v) glucose, 0.1 mM $CaCl_2$, 2 mM $MgSO_4 \cdot 7H_2O$ and 50 μg/L kanamycin at 37 °C with shaking (220 rpm) until OD_{600} reached a value between 0.6-0.8. Protein expression was induced by addition of 0.5 mM Isopropyl β-d-1-

thiogalactopyranoside (IPTG) and a series of different transition metal ion salts: $^{56}Fe^{2+}$ (250 μM $(NH_4)_2Fe(SO_4)_2$), Ni^{2+} (50 μM $NiSO_4$), Co^{2+} (200 μM $CoCl_2$) and Mn^{2+} (200 μM $MnCl_2$). Cell cultures were incubated at 18 °C with shaking (220 rpm) for 16-20 hours. Cultures were subsequently centrifuged at 8,000 x *g* for 20 min; cell pellets were flash frozen in liquid N_2 and stored at -80 °C prior to purification. Cell pellets were resuspended in the lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 300 mM sodium chloride (NaCl) and 10 mM imidazole, pH 7.5). The suspension was lysed using a QSonica sonicator (Newton, CT) (~ 30 min per 100 mL suspension) with the addition of 250 μM ammonium iron sulfate and 0.45 μg/L phenylmethylsulfonyl fluoride (PMSF) prior to and after sonication. Lysed cells were centrifuged at 22,000 x *g* for 30 min. The clarified lysate was loaded onto a Ni^{2+}-NTA immobilized affinity chromatography column (~100 mL resin per 500 mL lysate) equilibrated with the lysis buffer. The column was washed first with lysis buffer and then with wash buffer (50 mM HEPES, 20 mM imidazole and 200 mM NaCl, pH 7.5). The protein was eluted by washing with elution buffer (50 mM HEPES, 150 mM NaCl and 300 mM imidazole, pH 7.5). Fractions containing the protein of interest were pooled and concentrated at 3,500 x *g* using a 30 K Amicon Centrifugal Filter Unit (Tullagreen, Carrigtwohill Co. Cork, Ireland). The concentrated protein was then buffer exchanged in the storage buffer (50 mM HEPES, 300 mM NaCl and 10% (w/v) glycerol, pH 7.5) by dilution and concentration. The concentrated protein was loaded onto a size exclusion HiLoad™ 16/600 Superdex™ 200 pg column (GE Healthcare), which was equilibrated with the storage buffer. Fractions containing the pure protein of interest were pooled and further concentrated. Protein purity was estimated by SDS-PAGE with Coomassie staining, and protein concentration was determined using a molar absorption coefficient of 48,820 M^{-1} cm^{-1} at 280 nm

(https://web.expasy.org/protparam/). The iron content was determined by the ferrozine assay and ICP-AES (*vide infra*).[2]

Note on the purification of these enzymes.

The Fe-form of the enzyme may be purified anaerobically, however, the ferrous ions are pretty labile and the overall yield of metal-loaded active protein may be compromised. Perhaps a better route is the aerobic isolation of these types of proteins, following by treatment with a reducing agent under O_2-free conditions, removal of the reducing agent by desalting and storage of the protein in liquid nitrogen to prevent its oxidation.

Cleavage of SUMO by Ulp1. For the protease cleavage reactions, Ulp1 (~1 μg per mg of SUMO-SO3491) was incubated with SUMO-SO3491 for 16 to 20 hours on ice. The reaction mixture was then loaded onto a Ni^{2+}-NTA affinity chromatography column equilibrated with the lysis buffer. The cleaved protein was eluted by washing the column with the lysis buffer. SUMO, Ulp1 and uncleaved SUMO-SO3491 (which all contain an N-terminal His_6-tag) were eluted with the elution buffer. Fractions containing the untagged (native) SO3491 protein were pooled and concentrated at 3,500 x *g* using a 30 K Amicon Centrifugal Filter. The iron content was determined by the ferrozine assay.

Table S1. List of primers employed in this study.

Protein	Vector	Restriction Sites	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
WT	PDB.His.MBP	HindIII/XhoI	AATAAAGCTTATG AACGCGTTCCTG	ACAACTCGAGTCA GGTTTGAAA
D69A	pSUMO	EcoRI/HindIII	CTGATCCACGCG TGCGGTGTG	ACCCGCAAAATA CGCGAACTG
D283A	pSUMO	EcoRI/HindIII	CTGCTGCACGCG GTTGGCAAG	ACCCGCCACGTA CAGCAG

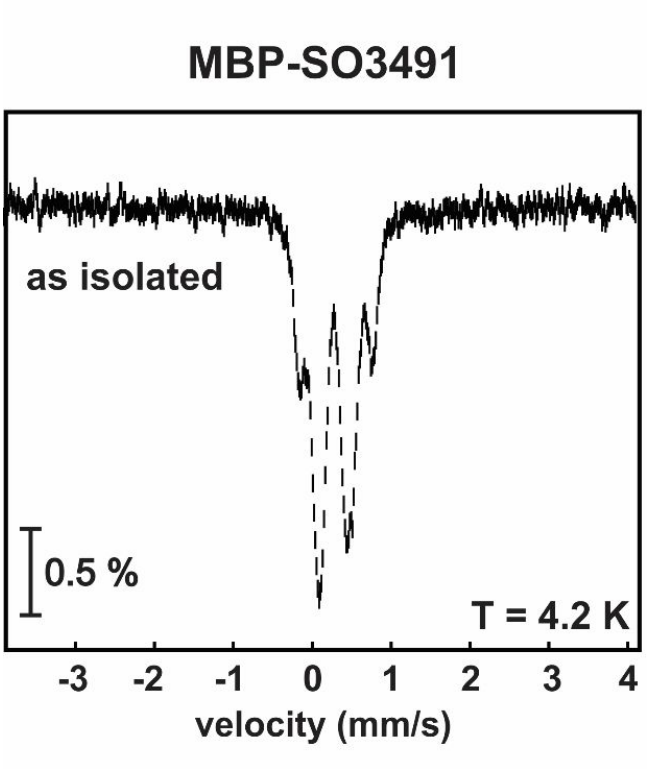

Figure S1. Mössbauer spectrum of the ^{57}Fe-labeled, aerobically isolated WT MBP-SO3491 recorded at 4.2 K and in the presence of a weak magnetic field (78 mT) applied parallel to the γ beam.

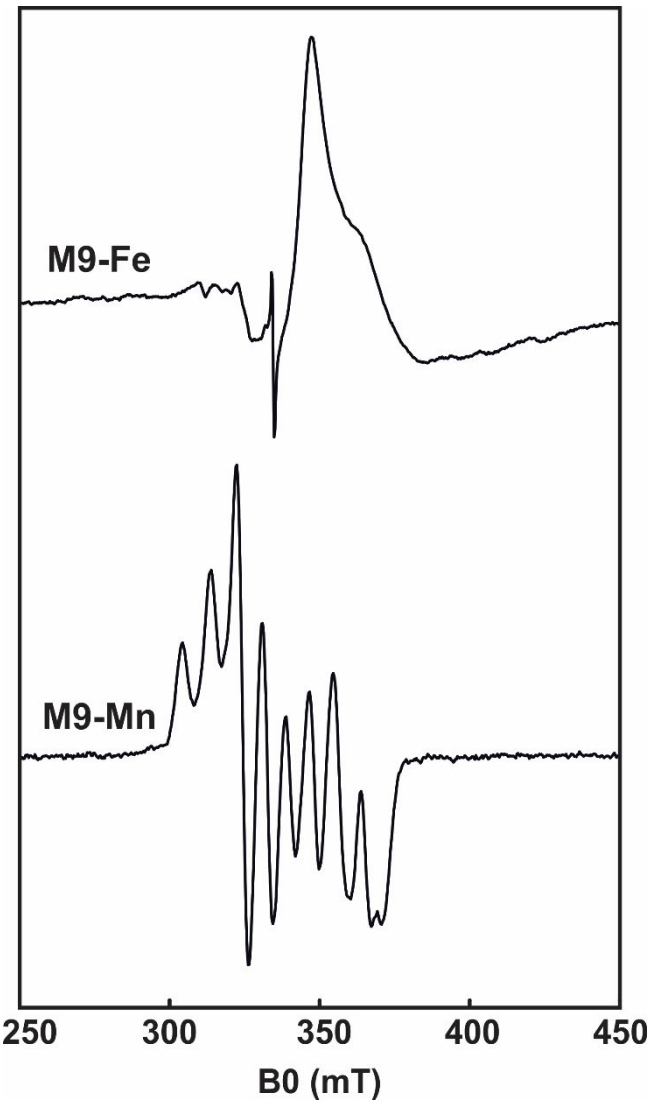

Figure S2. CW EPR spectra of the Fe- and Mn-containing WT MBP-SO3491. (Top) Fe-containing WT MBP-SO3491 reduced with 5 mM ascorbate under O_2-free conditions. (Bottom) Aerobically purified Mn-containing WT MBP-SO3491. Experimental conditions: microwave frequency = 9.36 GHz, microwave power = 2 mW, modulation amplitude = 1 mT, temperature = 10 K.

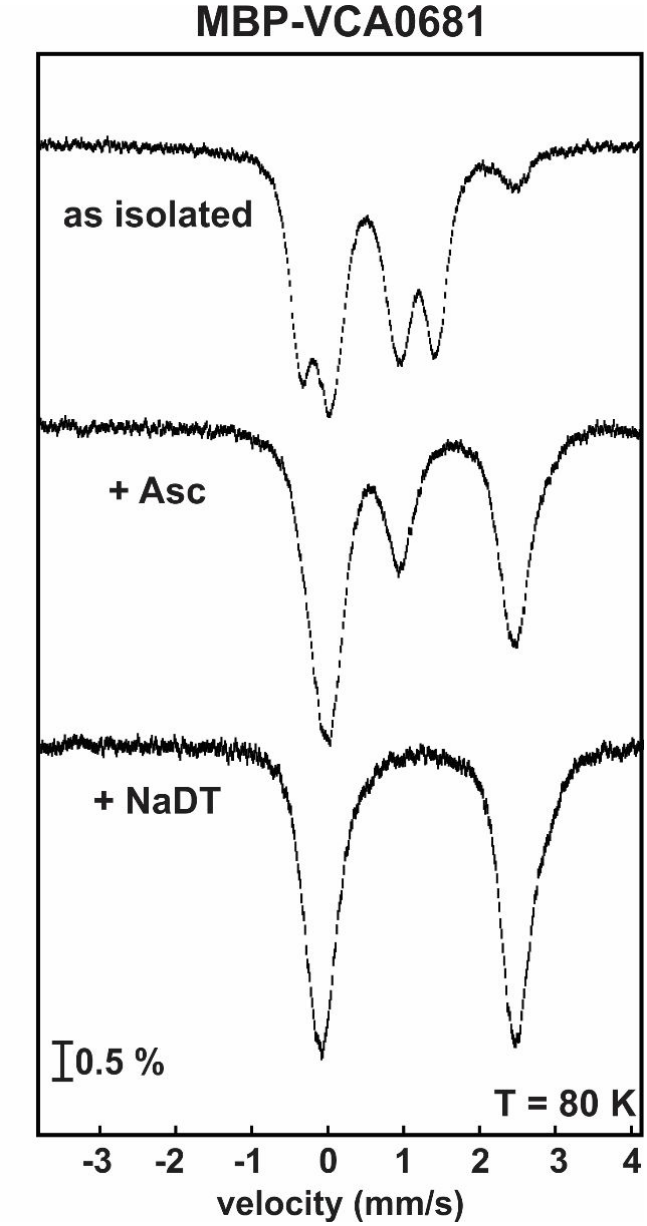

Figure S3. Mössbauer spectra of the WT MBP-VCA0681 at different redox states. High-temperature Mössbauer spectra of the WT MBP-VCA0681 aerobically isolated (top), reduced with 5 mM ascorbate (middle) and reduced with 10 mM dithionite (bottom). Experimental conditions: temperature = 80 K, B = 0 T.

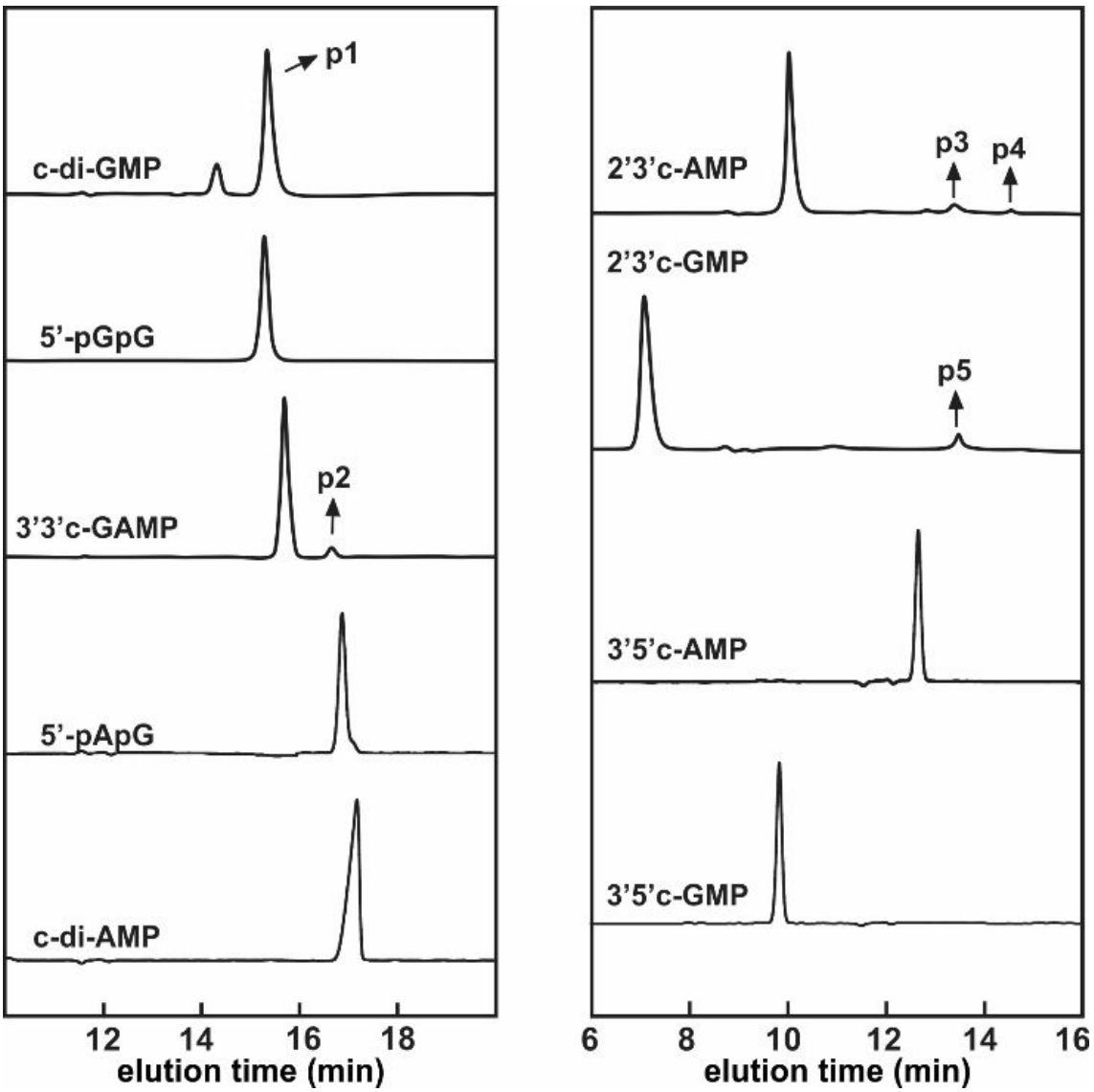

Figure S4. HPLC chromatograms monitoring hydrolysis of different nucleotides by the WT MBP-VCA0681 in end-point activity assays. X-axis is the elution time in minute and y-axis is the absorbance at 254 nm. All assays were performed under O_2-free conditions for 1 hour with a final concentration of protein 5 μM (diiron concentration) and 50 μM of substrate analogs. P1 corresponds to 5'-pGpG, p2 to 5'-pApG, p3 to 3'-AMP, p4 to 2'-AMP, and p5 to 3'-GMP.

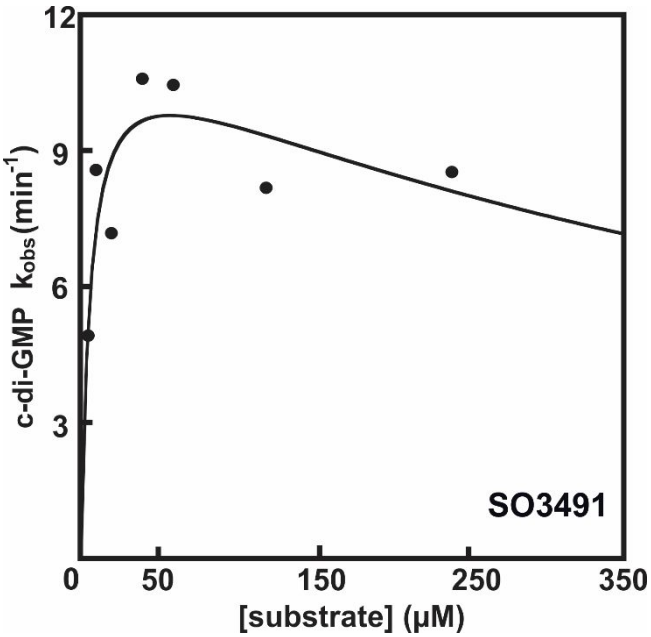

Figure S5. Steady-state kinetics of WT native SO3491 with c-di-GMP. Data are fitted with the Michaelis-Menten equation considering substrate/product inhibition using the equation: $\frac{v}{E_0} = \frac{k_{cat} * [S]}{K_M + [S] * (1 + \frac{[S]}{K_I})}$. An apprent K_M of 6 ± 3.7 μM, a catalytic constant of 0.2 ± 0.04 s^{-1} and an apparent inhibition dissociation constant K_I of 553 ± 529 μM were estimated.

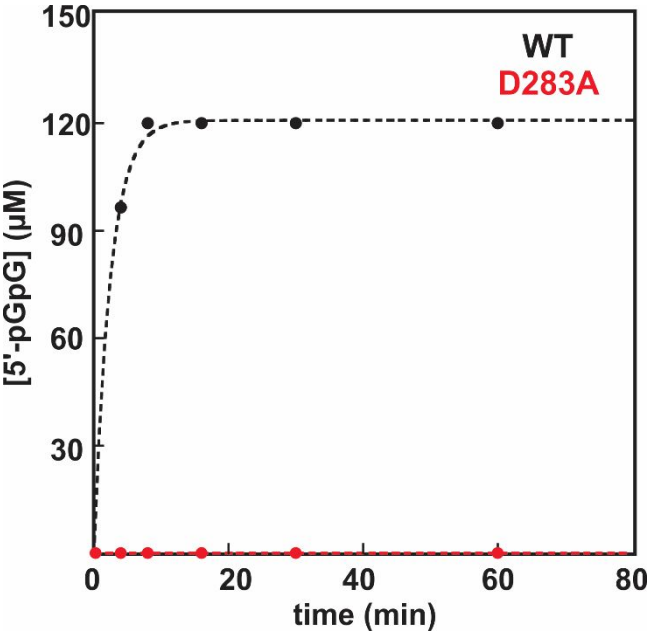

Figure S6. Time-dependent c-di-GMP hydrolysis of the WT and D283A SO3491. All assays were carried out with 1 μM cofactor concentration and 120 μM c-di-GMP at room temperature under O_2-free conditions.

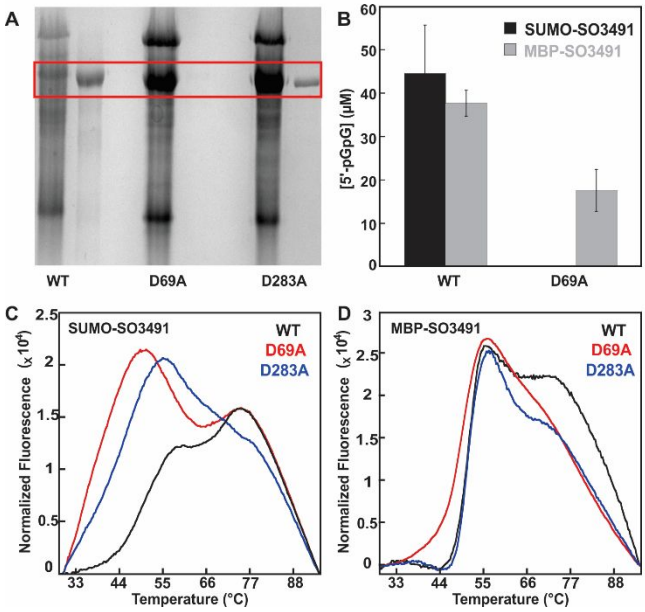

Figure S7. Purification, end-point c-di-GMP hydrolysis and thermostability of SO3491 fusions. (A) 12% SDS-PAGE gel of WT, D69A and D283A SUMO-SO3491. The left lane in each case correspond to the pellet and the right lane to the protein eluted from Ni-NTA. Bands representing the SUMO-SO3491 are boxed in red. (B) End-point c-di-GMP hydrolysis of 1 μM SUMO- and MBP-tagged WT, D69A (diiron concentration) with 60 μM c-di-GMP. Error bars represent standard errors. (C) Thermal shift assay of WT, D69A and D283A SUMO-SO3491. (D) Thermal shift assay of WT, D69A and D283A MBP-SO3491.

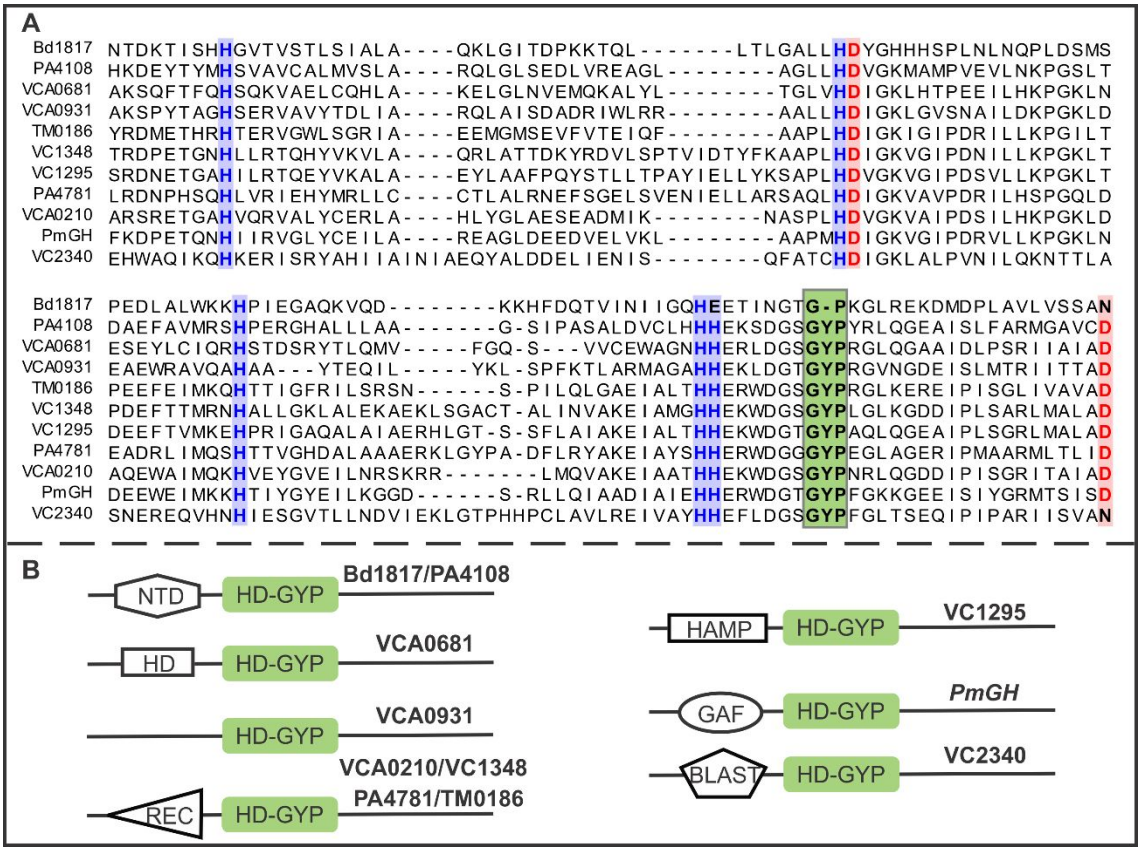

Figure S8. (A) Sequence alignment of the 11 HD-GYP sequences included in our phylogenetic analyses. The conserved protein residues of the ligand-binding [H…HD…H…HH…D] motif are highlighted in blue (histidine) and red (aspartate). The GYP motif, representative of the HD-GYP subfamily is highlighted in green. (B) Domain architecture of the 11 HD-GYP proteins examined.

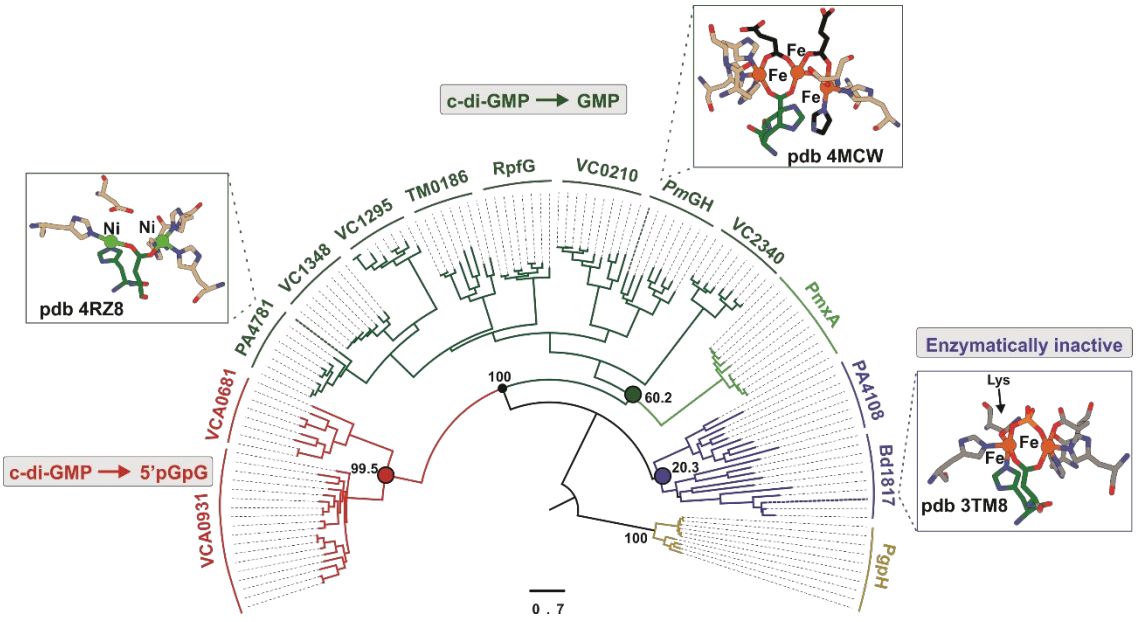

Figure S9. Rooted maximum-likelihood phylogenetic tree generated with 121 unique sequences of HD-GYP proteins and 10 sequences of the PgpH PDE subfamily as an outgroup. PgpH is a c-di-AMP specific phosphodiesterase (see main text). The tree was computed with the IQ-tree software using the LG+R5 model. The scale bar represents the number of substitutions per site. Sequences were aligned using the MAFFT software. Proteins segregated in three different major-branches are colored in red, green, and purple. The outgroup clade, PgpH, is colored in gold.

REFERENCES

(1) Miner, K. D., Klose, K. E., and Kurtz, D. M. (2013) An HD-GYP cyclic di-guanosine monophosphate phosphodiesterase with a non-heme diiron-carboxylate active site. *Biochemistry 52*, 5329–5331.
(2) Bollinger, JM, J. (1993) On the chemical mechanism of assembly of the tyrosyl radical-dinuclear iron cluster cofactor of E.coli ribonucleotide reductase. Massachusetts Institute of Technology.