## **Electronic Supplementary Information**

## 1. Methods

**Protein expression and purification**. Codon-optimized genes for *Bpe*GReg<sup>1</sup> and *Pcc*GCS (YP\_003018185.1; GenScript, codon optimization performed by GenScript using proprietary methods) were cloned into pET-20b (Novagen) using NdeI and XhoI restriction sites. The resulting plasmids (pET20-BpeGReg; pET20-PccGCS) were transformed into *Escherichia coli* Tuner (DE3) pLysS cells (Novagen) via electroporation and positive transformants were selected on LB medium containing ampicillin (100 µg mL<sup>-1</sup>) and chloramphenicol (30 µg mL<sup>-1</sup>). Expression strains were grown in yeast extract medium (YM) to late exponential phase (OD<sub>600</sub> = 0.8) at 37 °C. Cells were then induced with IPTG (1.0 mM) and temperature lowered to 25 °C. Cells were allowed to express DGC proteins for 6 hours (to minimize the amount of endogenous c-di-GMP bound to resulting overexpressed proteins). Cells were harvested by centrifugation (3,500 x g, 4 °C, 20 min) and resulting cell pellets were immediately resuspended in Buffer A (50 mM Tris, 50 mM NaCl, 1 mM DTT, 20 mM imidazole, pH 7.4).

Cell pellets were homogenized using a homogenizer (Avestin, Inc.) and resulting lysates were centrifuged at 130,000 x g in a Beckman Optima L-90X ultracentrifuge at 4 °C for 1 hour. All subsequent purification steps were performed at 4 °C. Supernatants were applied to a pre-equilibrated HisPur Ni-column (Fisher Scientific) and proteins were eluted with buffer B (buffer A with 250 mM imidazole, pH 7.4). Purified proteins were desalted using a S200 gel filtration column (GE Healthcare) into buffer C (50 mM Tris, 50 mM NaCl, 1 mM DTT, 5% Glycerol (v/v), pH 7.0). Proteins were collected and concentrated via ultrafiltration (YM-10, 10 kDa MWCO filter, Millipore), aliquoted, flash frozen, and stored at -80 °C until use.

Phosphodiesterase  $EcDosP^2$  was purified in an analogous manner to the GCSs, with the exception that it was expressed from pET-28a, so cells were grown in the presence of kanamycin (30 µg mL<sup>-1</sup>).

## PccGCS optimized gene sequence.

CGGTACGCCGCTGAGTTCCATTCAGGATCTGAGTGACTCCGAATTTGGCCTGT GGTTCAACCATAAAGGTAAGTCATCGTTCAGCAATATCCAAGGCATTCGTAC CATCGCGTCTATTATCACCGAAACGGATGAATATATTCGCGGTTACTCTACCA CGGCCCTGCTGACCCAGCAAGATTATGCACCGCTGCTGAAAGCTGTGCGTAG TAAAATTTACAAGATCAACATGCTGCTGGGGCAGTCTGTTTGATGAAGTTCAG AAACTGGAATCCGGCAAGGACACCCTGACGCTGCTGCTGAATCGTCGCTTCC TGCCGACCATCCTGCGCCATGAAATTTCACTGGCGATGCACAGCTCTACCCCG CTGTCGATTGCCATGATCGATATTGACCATTTTAAAACCGTGAACGATACGTA TGGCCACGCAGCTGGTGACAATATTCTGAAACGTATCGCGGAAGTTCTGTAT GAAAGCACCCGTAACTCTGATTATGTCTTTCGCTACGGCGGTGAAGAATTTAT GATTGTCCTGATCGAAACCCCGAAAGTGGCGGCCCATACGATTATCGAACGT CTGCGCAAAAAGATCCAGGATCATCCGATTCACCTGCAAAATGGCGAAAGCA TTACCATGACGATCTTGCGGGCATTGCCGTTTATAGCGGTCACCCGGATAAC GAATGTCTGATCAAGGCAGCTGATGACGCACTGTATCAGGCGAAAGCCAACG GTCGTAATCGCATTGAATACGCTCAAGAAGAATAA

**Blue-Native PAGE.** Blue Native PAGE was performed using the NativePAGE Bis-Tris gel system (Life Technologies). Briefly, protein concentrations were quantified and loaded onto NativePAGE Novex 3-12% Bis-Tris precast gels at multiple concentrations. BN-PAGE sample buffer contained 0.002% Coomassie-G250 additive (w/v). Gels were run at constant voltage (160V) until the dye front reached ~90% of the gel length and then were stained using the Pierce Silver Stain Kit (Thermo-Scientific) according to the manufacturers instructions. Gels were imaged and analyzed with ImageJ software. For comparison, protein markers were run alongside and consisted of the following proteins in native conformations: IgM Hexamer (1236 kDa), IgM Pentamer (1048 kDa), Apoferritin band 1 (720 kDa), Apoferritin band 2 (480 kDa), B-phycoerythrin (242 kDa), Lactate Dehydrogenase (146 kDa), Bovine Serum Albumin (66 kDa), Soybean Trypsin Inhibitor (20 kDa).

**Electronic spectroscopy.** All spectra were recorded on an Agilent Cary 100 with Peltier accessory. Preparation of complexes was carried out as previously described except that the proteins were prepared in Buffer C.<sup>3-5</sup>

**HPLC-based c-di-GMP detection.** Measurement of c-di-GMP production by HPLC was performed as previously described.<sup>1</sup>

Analytical gel filtration. GCS oligomers were detected via size exclusion chromatography using an Agilent 1200 infinity system with a Sepax SEC-300 (7.8 mm X 300 mm, 300Å) and diode array detector (simultaneous detection at 214, 416, and 431 nm). Proteins were reduced in an anaerobic chamber and then allowed to bind  $O_2$  following mixing with aerobic buffer before injection onto SEC-300 column. The mobile phase for all experiments consisted of 150 mM sodium phosphate, pH 7.0, with 1 mM DTT. Spectra were collected for each peak during the SEC run to confirm that the heme remained in the Fe<sup>II</sup>-O<sub>2</sub> ligation state. Globular proteins (Sigma-Aldrich) consisting of thyroglobulin (669 kDa), ferritin (443 kDa),  $\beta$ -amylase (200 kDa), alcohol

dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and conalbumin (29 kDa) were used as molecular weight standards for calibration curves.

**Enzyme kinetics.** Prior to diguanylate cyclase kinetic assays, the proteins were reduced and various complexes formed as previously described.<sup>3-5</sup> The ligation/oxidation state of the heme was determined by UV-vis spectroscopy before each enzyme assay. All Fe<sup>II</sup> unligated kinetics were measured in an anaerobic chamber. The EnzChek pyrophosphate kit (Life Technologies) was used according to the manufacturers instructions with the exception that GCS proteins (BpeGReg, PccGCS) and PDE (EcDosP) were added and the reactions were initiated with varying concentrations of GTP. Briefly, the EnzChek kit monitors production of pyrophosphate from the enzymatic conversion of GTP to c-di-GMP. The kit contains an inorganic pyrophosphatase (IP) and nucleotide phosphorylase (NP). PP<sub>i</sub> is converted to P<sub>i</sub> via IP and the resulting P<sub>i</sub> is used to enzymatically convert 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. The enzymatic conversion of MESG results in a shift in absorbance maximum from 330 nm to 360 nm. A<sub>360</sub> readings were monitored every 30 seconds for 180 minutes.

Assays were performed in triplicate in 96 well plates containing 4 protein concentrations (0.5-3uM) and 8 GTP concentrations (0-500uM). EcDosP was included at 3-molar excess to eliminate inhibition of cyclase activity by the produced c-di-GMP.<sup>1</sup> Additional molar equivalents of EcDosP did not result in changes to the rate. Plates were monitored using an Epoch plate reader and Gen5 software (Biotek). The entire plate assay (including triplicates) was repeated at least twice to account for day to day and protein batch variability. Assays of Fe<sup>II</sup>-CO and Fe<sup>II</sup>-NO kinetics were performed in anaerobic cuvettes and quantified using an Agilent Cary 100. Subsequent analyses to determine enzymatic rates were performed using Igor Pro (Wavemetrics).

 $O_2$  dissociation rate.  $O_2$  dissociation rates were performed as previously described with the following modifications.<sup>3, 5</sup> Protein for  $O_2$  dissociation rates and dithionite traps (1-10 mM) were prepared in Buffer C. Saturating CO was not used as part of the trap. The dissociation of  $O_2$  from the heme was monitored using an SX20 equipped with a diode array detector and fit globally using Pro-KII (Applied Photophysics). Additional fitting analysis was performed using Igor Pro (Wavemetrics).

**Figure S1.** Sequence alignment of the globin coupled sensors from *E. coli* (*Ec*DosC), *P. carotovorum* (*Pcc*GCS), *S. putrefaciens* (*Sp*DosD) *B. pertussis* (*Bpe*GReg), *D. psychrophila* (*Dp*HemDGC).



**Figure S2.** MALDI mass spectra of *Bpe*GReg (A. calculated = 53753.82, observed = 53828) and *Pcc*GCS (B. calculated = 54883.01, observed = 54977).



**Figure S3.** Representative data from which Michaelis-Menten parameters were calculated for *Bpe*GReg (A. rate vs. [GTP]; B.  $v_{max}$  vs. [*Bpe*GReg]).





**Figure S4.** Blue-Native PAGE of *Bpe*GReg and *Pcc*GCS (concentration decreasing from left to right on each gel).

Figure S5. Representative re-equilibration of *Pcc*GCS tetramer over time.



**Figure S6.** Stopped flow kinetics. Representative overlayed stopped flow spectra for the as purified oligomeric mixtures of *Bpe*GReg (A.) and *Pcc*GCS (D.), as well as comparison of mono-exponential and bi-exponential fits for representative experiments for *Bpe*GReg (B. and C., respectively) and *Pcc*GCS (E. and F., respectively) are shown. Raw data at 436 nm is shown in red and the calculated fit in black. The fit residuals (difference between the calculated fit and raw data) are shown in black above each plot.



Protein	Oligomer	$k_1 (s^{-1})$	$k_2 (s^{-1})$	% <i>k</i> 1	% k2
<i>Bpe</i> GReg	As purified	$0.82 \pm 0.01$	$6.30 \pm 0.11$	$39.3 \pm 2.2$	$60.7 \pm 2.2$
	Dimer	$1.33 \pm 0.07$	$6.16 \pm 0.97$	$40.7 \pm 2.3$	$59.3 \pm 2.3$
	Tetramer	$1.23 \pm 0.01$	$7.50 \pm 0.06$	$36.2\pm0.9$	$63.8\pm0.9$
PccGCS	As purified	$0.56 \pm 0.01$	$3.87\pm0.08$	$56.1 \pm 1.0$	$44.0\pm1.9$
	Dimer	$0.668 \pm 0.018$	$4.68 \pm 0.25$	$56.1 \pm 0.8$	$43.9\pm0.8$
	Tetramer	$0.641 \pm 0.003$	$3.98 \pm 0.03$	$56.3 \pm 1.7$	$43.7 \pm 1.7$

Table S1. Oligomer-dependent O<sub>2</sub> dissociation rates and percentages.

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