Supplementary Material (ESI) for Molecular BioSystems

Supplemental Materials

Experimental Methods

Materials and general methods
Unless otherwise noted, all reagents were purchased in their highest available purity and used as received.

Multiple sequence alignment
ClustalW2 was used to generate multiple sequence alignments of GGDEF and EAL domains from GGDEF/EAL hybrid proteins. Proteins in the alignment are as follow: *Sw*DGC (YP_001761121), from *Shewanella woodyi*; *Ax*DGC3 (AAC61689), from *Acetobacter xylinum*;[1] *St*GcpF (NP_462298), from *Salmonella typhimurium*;[2] *Ax*PDE3 (AAC61688), from *Acetobacter xylinum*;[1] *Cc*PDE (AAK25358), from *Caulobacter crescentus*;[3] *Ec*YciR (AAN80222), from *Escherichia coli*;[4] *Ms*DGC1 (YP_886551), from *Mycobacterium smegmatis*;[5] *Rs*BphG1 (AAL50635), from *Rhodobacter sphaeroides*;[6] and *Vp*ScrC (AAK08640), from *Vibrio parahaemolyticus*.[7] ClustalW2 was used to generate multiple sequence alignments of PAS domains. Proteins in the alignment are as follow: *Ns*HNOBA (BAB73978), from *Nostoc* sp.;[8] *Ax*DGC2 (AAC61687), from *Acetobacter xylinum*;[9] *Ax*PDEA1 (BAD36772), from *Acetobacter xylinum*;[8] and *Vp*ScrG (AAO61794), from *Vibrio parahaemolyticus.*[7]

Construction of expression plasmids
Genomic DNA was extracted from *Shewanella woodyi* (ATCC 51908) using the Wizard® kit from Promega. PCR was used to amplify Swoo_2750 from *Shewanella woodyi* genomic DNA using the PfuUltra® AD polymerase (Stratagene). The upstream primer was 5'-ggaattcccatatgagtgcacttgaggacagagaacaagtttttctg-3'. The downstream primer was 5'-ggaattccctcgagtttagcgggtaaagagatacagcgctgcatatg-3'. Upstream and downstream primers contained *Nde*I and *Xho*I (New England Biolabs) restriction sites, respectively. All amplified PCR products were cloned into pET-28b (Novagen) in frame with an N-terminal $(His)_6$ tag followed by a stop codon and sequenced (Stony Brook sequencing core). Mutagenesis was carried out using the QuikChange® protocol from Stratagene. Mutagenesis for *Sw*GGAAF was carried out in two steps, serially replacing each acid residue with an alanine. The primers used were 5'-gttaacacaatagagaactcagctccccccaacctggcaa-3' and its reverse complement followed by 5'-gttaacacaatagagaacgcagctccccccaacctggcaa-3' and its reverse complement. The mutagenic primers used to create the *Sw*AAL plasmid were 5'-ccaacggatcaaggctgcagcaaaggtcacttc-3' and its reverse complement.

Protein expression and purification
A single colony of *Sw*DGC transformed *E. coli* Tuner (DE3) plysS cells were grown in YT media (10 g/L yeast extract and 15 g/L tryptone) with chloramphenicol (34 µg/mL) and kanamycin (10 µg/mL) at 37 ˚C and 250 rpm. When the optical density of cells reached ~0.5 at 600 nm, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration 0.1 mM, and the temperature was lowered to 18 ˚C. Expression took place for 12 hours. The cells were then collected by centrifugation and resuspended in buffer A (50 mM sodium phosphate, pH = 8.0, 300 mM sodium chloride). The cells were then lysed by sonication, and centrifuged at 18,000 rpm for 2 hours. The supernatant was loaded onto a Ni-NTA column (GE Life Sciences) pre-equilibrated with buffer A. The column was washed with buffer A containing increasing concentrations of imidazole (20, 50, and 100 mM). The protein was then eluted with buffer A containing 250 mM immidazole. The protein fractions were combined and desalted into buffer B (50 mM Tris, pH = 7.5, 50 mM sodium chloride, 1 mM DTT, and 5% glycerol) using a PD-10 column (GE Life Sciences). Temperature was maintained at 4 ˚C during the entire purification process to minimize proteolysis. Protein aliquots were flash frozen and stored at -80 ˚C. Purity was checked using 12% SDS-PAGE.

Trypsin digestion and mass spectrometery analysis
Trypsin (0.5 mg/mL; Promega) was added at a 1:20 molar ratio to protein samples (20 µM final concentration) and the mixtures were incubated over night at 37 ˚C. α-Cyano-4-hydroxycinnamic acid (Sigma-Aldrich) was dissolved in 50% acetonitrile as the matrix and 1:1, 2:1, 5:1, 10:1, 20:1 matrix to peptide ratio samples were prepared. Peptide samples (1 µL) were spotted on a MTP 384 massive target T plate (Bruker) for analysis by matrix-assisted laser desorption/ionization - time of filght (MALDI-TOF) mass spectrometery on an Autoflex II (Bruker) mass spectrometer. FlexControl and FlexAnalysis (Bruker) were used for data collection and analysis.

Enzymatic assay
This assay was based on one described previously.[10] *Sw*DGC wild type and mutants (10 µM) were incubated at 37 ˚C in buffer (75 mM Tris·HCl, pH = 7.5, 250 mM NaCl, 25 mM KCl, 200 µM GTP and 10 mM $MgCl_2$) for 0 – 12 hours. Aliquots (40 µL) were then heat denatured at 95 °C for 5 min, followed by centrifugation for 10 min at 14,000 rpm and filtered by Centriprep® filters (Millipore). Supernatant was analyzed by HPLC using reverse phase C18 column (Shimazu) with 10% methanol in buffer (50mM triethylammonium acetate, pH = 6.0). Peaks were collected and identified by comparison of retention times to standard nucleotides (c-di-GMP and pGpG [Biolog]) and MALDI-TOF mass spectroscopy. The spectrum settings were used as previously reported.[11] Each experiment was repeated at least three times.

Congo red plate assay

This assay took place as previously reported.[12] Briefly, agar plates containing 5 g/L yeast extract, 10 g/L tryptone, and 15 g/L BactoAgar and supplemented with 50 µg/mL Congo red (CR) dye (Sigma-Aldrich) and IPTG (0.00, 0.05, and 0.10 mM) were prepared. Cell cultures of *Sw*DGC constructs transformed into Tuner (DE3) plysS cells were shaken at 250 rpm in YT media at 37 °C until an OD of ~0.5 before being streaked onto the plates. Plates were incubated at 30 °C for 12 hours followed by incubation at room temperature for an additional 24 hours. The appearance of the bacterial growth was visually assessed for varying CR intensities. Each experiment was repeated at least three times.

Congo red liquid-binding assay

This assay took place as previous reported.[13] Briefly, media containing 5 g/L yeast extract, 10 g/L tryptone, and 150 mM phosphate buffer (pH = 7.0) was supplemented with 25 µg/mL CR. Overnight cultures were diluted 100-fold in fresh media and grown to an OD of ~0.5 at 37 °C and 250 rpm. 1 mL aliquots of each culture were pipetted into sterile plastic culture tubes stoppered with styrofoam. Each culture was supplemented with IPTG (0.00, 0.05, and 0.10 mM) before being shaken at room temperature and at 250 rpm for 14 hours. After incubation, a 200,000-fold dilution was made from each tube and plated in order to calculate colony forming units (CFU). Plates were incubated at 30 °C for 12-16 hours. The rest of each culture was centrifuged at 14,000 rpm for 15 minutes and 100 µL of the supernatant was pipetted into a 96-well microtiter plate for absorbance measurements at 500 nm using Victor X microplate reader (Perkin Elmer). Each experiment was repeated at least three times.

Supplemental Figures

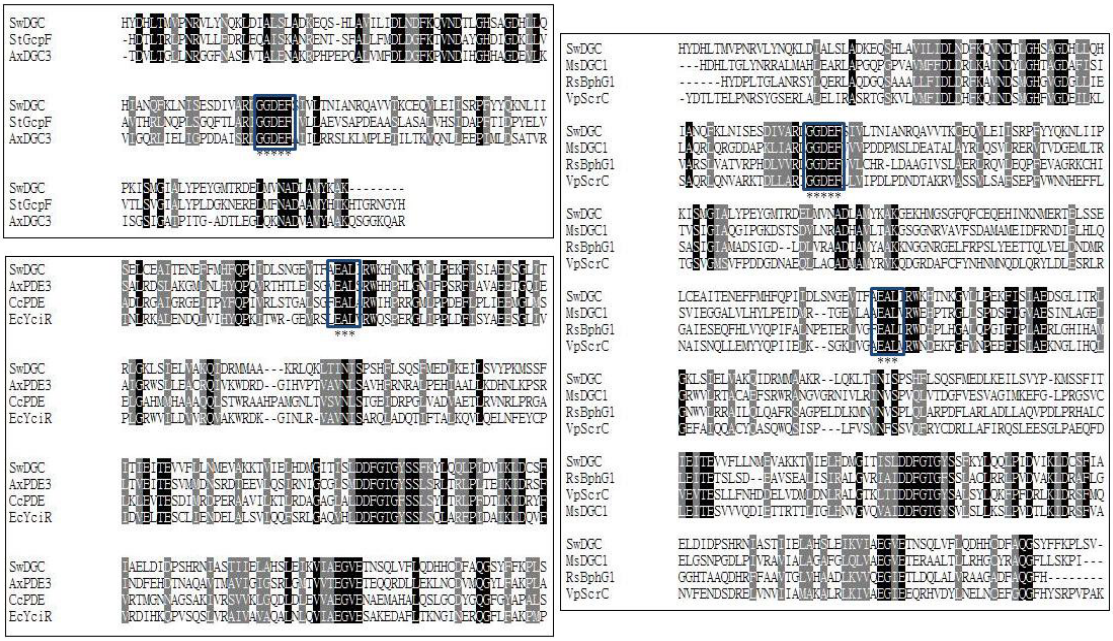

Figure S1. Multiple sequence alignments of proteins containing both GGDEF and EAL domains. Top left: Alignment of the GGDEF domain only from proteins containing active GGDEF domains and inactive EAL domains. The GGDEF domain from *Sw*DGC has an average of a 32% identity score with other active GGDEF domains in GGDEF/EAL hybrid proteins with inactive EAL domains. Bottom left: Alignment of the EAL domain only from proteins containing inactive GGDEF domains and active EAL domains. The EAL domain from *Sw*DGC has an average of a 33% identity score with other active EAL domains in GGDEF/EAL hybrid proteins with inactive GGDEF domains. *Sw*DGC has the conserved DDFGTG sequence that is thought to be necessary for phospodiesterase activity. Right: Alignment of both the GGDEF and EAL domains from proteins with active GGDEF and active EAL domains. *Sw*DGC has an average of a 31% identity score across both domains with GGDEF/EAL hybrid proteins with dual activity. Black bars: conserved amino acids; grey bars: similar amino acids; the * indicates the "GGDEF" & "EAL" sequences for which the domains are named.

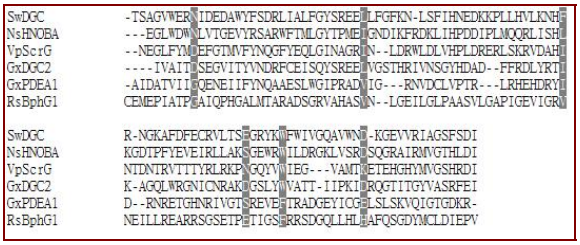

Figure S2. Multiple sequence alignments of PAS domains. The top sequence is from *Sw*DGC, the second sequence is a PAS domain from the H-NOBA family, PAS domains that are thought to mediate protein-protein interactions between sensory and enzymatic domains,[14] and the final four sequences are PAS domains found in the same polypeptide sequence as GGDEF or EAL domains and that are known to regulate their enzymatic activity. *Sw*DGC has an average of a 13% identity score with those PAS domains; *Sw*DGC has a 25% identity score with the H-NOBA domain and 18%, 3% and 16% identity scores with the sensory PAS domains. Grey bars: similar amino acids.

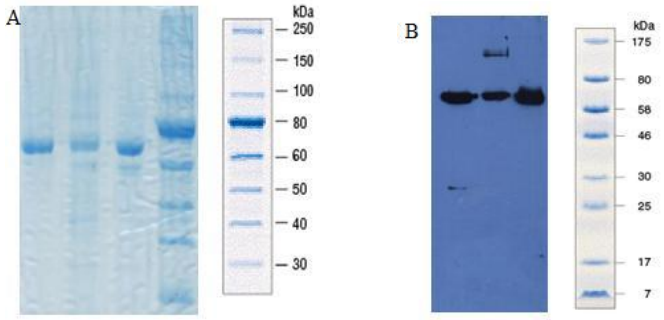

Figure S3. *Sw*DGC purification and detection. (A) Coomassie stained SDS-PAGE. Left to right: *Sw*GGAAF, *Sw*AAL, *Sw*DGC, broad range protein ladder (New England Biolabs P7703S). (B) Western blot of SDS-PAGE using an antibody to the $(His)_6$ tag. Left to right: *Sw*GGAAF, *Sw*AAL, *Sw*DGC, pre-stained ladder (New England Biolabs P7708S).

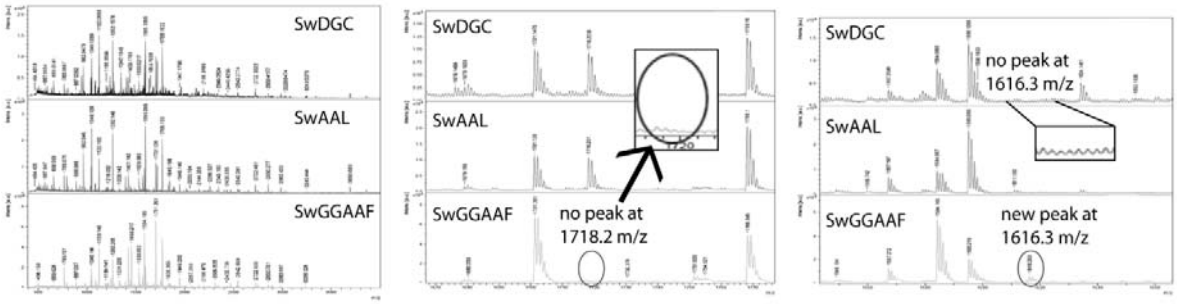

Figure S4. Mass spectroscopy of trypsin digested proteins. Peptides totalling 79% coverage of each protein were found. Top to bottom: *Sw*DGC, *Sw*AAL, and *Sw*GGAAF. A mass shift from 1718 m/z to 1616 m/z indicated the success of the GGDEF to GGAAF mutation. Similarly, the success of the EAL to AAL mutation was demonstrated in a peak shift from 858 m/z to 1080 m/z in a chymotrypsin digest (data not shown).

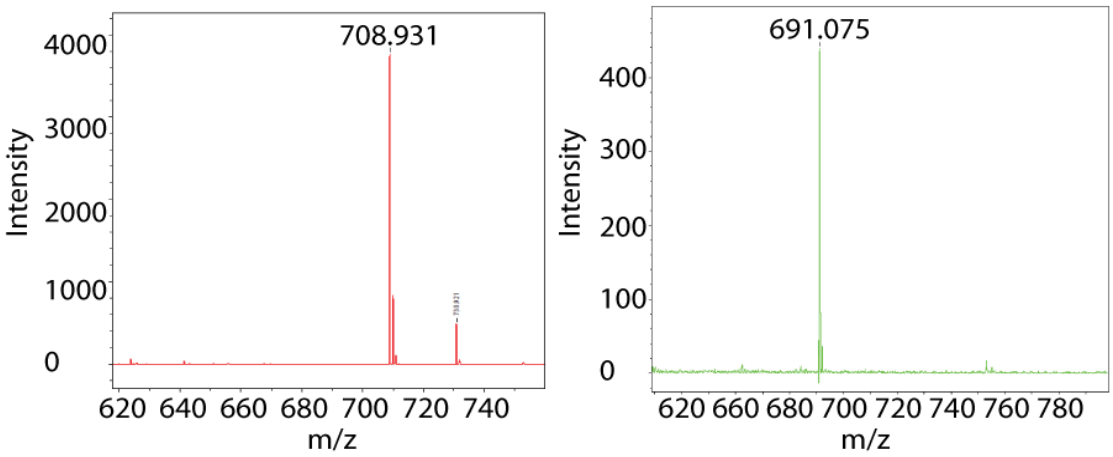

Figure S5. Identification of HPLC products by MALDI. Left: the product collected at 13 min from the HPLC; right: the product collected at 28 min from the HPLC. The correct molecular weights were observed for pGpG (708 g/mol) and c-di-GMP (690 g/mol) with a mass difference of 1 proton.

Supplementary References

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