SUPPLEMENTAL INFORMATION

Materials and Methods

Strains and Media. Bacterial strains and plasmids used in this study are listed in Table S4. Primers used to generate these strains and plasmids are listed in Table S5. Gentamycin (Gm) was used at concentrations of 30 ug/ml in LB agar and 15 ug/ml in liquid LB for *P. fluorescens*, and at 10 ug/ml for all *E. coli* growth conditions. Kanamycin (Km) and carbenicillin (Cb) were used at concentrations of 50 ug/ml for culturing *E. coli.* All genomic deletions were achieved using the pMQ80 vector by recombination (1). Constructs on the pMQ72 and pMQ80 were cloned in the *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen) using previously described techniques (1). Biofilm experiments and subculturing of *P. fluorescens* was conducted with the minimal medium K10T-1 as described previously (2).

P. aeruginosa PA14 cells were transformed as follows: 1 ml of overnight, LB-grown *P. aeruginosa* PA14 culture was washed twice with 300 mM sucrose, then resuspended in 100 ml of 300 mM sucrose, and 40 ml of this cell suspension was used for transformation. *P. fluorescens* was transformed in a similar manner, but washes began with 2 ml of overnight culture and was washed twice with 300 mM sucrose.

The pUT18 and pKNT25 expression vectors used in the bacterial two-hybrid assay were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For all subculturing, single colonies of *P. fluorescens* strains were grown in LB overnight with the appropriate antibiotic. After 16 hours, cultures were inoculated 1:75 into 45 ml of K10T-1 in a 250 ml flask at 230 rpm at 30ºC for 6 hours. Cells for this subculture were used in the fractionation and co-precipitation experiments. Error bars in biofilm and bacterial two-hybrid assays represent standard deviation.

Construction of Point Mutations. Point mutations were constructed using a modified point

mutagenesis technique. Briefly, forward and reverse primers with the mutation of interest were used separately to amplify the parental vector with Phusion polymerase. After four cycles of amplification, products from the forward and reverse primer were combined and amplification continued for 18 cycles. Unmodified parental plasmid DNA from *E. coli* was digested using the DpnI restriction enzyme for four hours at 37ºC, and the products were transformed into *E. coli*. Mutations were verified by sequencing.

Bacterial Two-Hybrid Assay and Screen. Genes coding for proteins to be tested for interaction in this assay were cloned into the expression vectors pUT18 and pKNT25 by ligation (3). Plates were allowed to incubate at 30ºC for 24 hours at which time the colonies were scraped off the plates in 1 ml of M63 minimal medium. When mutant variants with interaction defects were being assayed, the colonies were grown for 17 hours instead of 24 hrs. Cells were scraped off of plates in M63 minimal medium. The resulting cell suspension was diluted to an OD_{600} value of approximately 0.5. 100 µl of the samples were added to 900 µl of Z-Buffer (60mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, pH adjusted to 7.0). Samples were solubilized with 50 µl chloroform and 25 µl 0.1% SDS followed by 10 seconds of vortexing and incubation at 30º C for 5 minutes. 200 µl ortho-Nitrophenyl-β-galactoside (ONPG – 4 mg/ml in Z-buffer) was added and samples were incubated at 30ºC for 5 minutes. The reaction was stopped with 500 µl of 1M Na_2CO_3 and absorbance was read at 420 and 550 nm. β-galactosidase activity was calculated where Miller Units = $[(OD_{420} - 1.75*OD_{550})/(\text{time in minutes}*OD_{600}*\text{volume of scrapped cultured})]*1000$. All mutant constructs were confirmed by sequencing.

When the bacterial two-hybrid system was used to screen mutants for interaction defects, the same procedure was followed, except 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to the plates, which were observed for pale blue or white

colonies after 17 hours compared to the blue colony phenotype indicating the interaction of the wild-type proteins. These colonies were cultured in selective LB, plasmid DNA isolated, and the plasmids sequenced to determine the site of mutagenesis.

Fractionation of Cells. Sub-cellular fractionation was performed as described previously (4). Briefly, cells were lysed by French press and the whole cell lysate was centrifuged at 100,000 x *g* for 1 hr to separate the cytoplasmic fraction from the total membrane fraction. The total membrane fraction was incubated for 20 min at room temperature in 2% sarkosyl detergent, and centrifuged as above to separate the inner from the outer membrane fraction. Each fraction was stored at -80ºC in 10% glycerol.

Dot Blot. Dot blots were conducted by growing *P. fluorescens* in 16hr LB cultures followed by sub-culturing in K10 minimal medium for 6 hours by diluting the overnight 1:75. Samples were normalized to the wild-type strain and 5 ul were blotted onto nitrocellulose paper and dried overnight.

Western Blotting. 25 ul of each SDS-treated sample was loaded onto a TGX protean gel (Bio-Rad). GcbC, LapD, and LapA were analyzed on 7.5%, 10%, and 4-12% acrylamide gels, respectively. The OprF and SadC homologues of the *P. aeruginosa* proteins were analyzed on a 10% gel, probed with antibodies raised to the *P. aeruginosa* proteins (5) and served as fractionation controls. Blots were blocked for an hour at room temperature in a 3% BSA solution, or a 2% milk solution in the case of OprF.

Protein Expression and Purification. A DNA fragment encoding the GGDEF domain of *P. fluorescens* GcbC (residues 339-501) was amplified from *P. fluorescens* genomic DNA by PCR and cloned into the pET-21a bacterial expression vector (Novagen), which adds a C-terminal His_6-affinity tag. Native $GcbC^{GGDEF}$ protein was overexpressed in *E. coli* BL21 cells. Cultures were grown in TB medium, supplemented with 100 μg/ml ampicillin at 37° C with shaking. When cultures reached an optical density at 600 nm (OD_{600}) of ~0.8, the temperature was

reduced to 18°C and protein expression was induced by addition of 0.5 mM IPTG. GcbC^{GGDEF} protein was expressed for 16 hours, after which cells were harvested by centrifugation, resuspended in NiNTA buffer A (25 mM Tris-HCl, pH 8.5; 500 mM NaCl; 20 mM Imidazole), and flash frozen in liquid nitrogen.

For purification of heterologously expressed protein, frozen cell suspensions were thawed and cells were lysed by sonication. After centrifugation, clarified lysates were incubated with NiNTA Superflow Resin (Qiagen) that was equilibrated with NiNTA buffer A. The resin was washed with 20 column volumes of buffer A, followed by elution of protein using 3 column volumes of NiNTA buffer B (NiNTA buffer A supplemented with 300 mM Imidazole). Eluted proteins were subjected to size exclusion chromatography on a Superdex 200 column (GE Healthcare Life Science) that was equilibrated in gel filtration buffer (25 mM Tris-HCl [pH 7.5], 250 mM NaCl). Purified proteins were concentrated using 10 KDa Amicon Ultra filters (Millipore), flash frozen in liquid nitrogen and stored at -80°C.

Crystal Structure Software. Data reduction was performed with the software package HKL2000 (6). The initial structure was solved by Molecular Replacement using the software package Phenix (7), and the crystal structure of the isolated GGDEF domain *P. aeruginosa* WspR (pdb 3I5B) as the search model. Refinement in Phenix and COOT (8) yielded the final models. Structural illustrations were made in Pymol (Schrödinger, LLC). Crystallographic software was made available through SBGrid (9). Data collection and refinement statistics are summarized in Supplementary Table 2.

Supplemental References

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