Supplemental Experimental Procedures

Bacterial strains and media

Escherichia coli strains were grown in M9L medium (M9 minimal medium supplemented with 5% LB (v/v) and 0.4% glycerol) at 37°C, unless otherwise indicated. To induce expression from the P_{BAD} and P_{lac} promoters, media was supplemented with 0.2% arabinose or 100 μM IPTG, respectively. All toxins were cloned into the SacI and HindIII sites of the arabinose-inducible pBAD33 vector, and all antitoxins were cloned into the SacI and HindIII sites of the IPTG-inducible pEXT20 vector. Toxin and antitoxin plasmids were cotransformed into *E. coli* TOP10 cells and plated on LB medium with 0.4% glucose and appropriate antibiotics. Single colonies were grown to saturation overnight in M9L medium with 0.4% glucose and antibiotics. The following morning, cultures were serially diluted and spotted onto M9L plates supplemented with antibiotics and 0.4% glucose, 0.2% arabinose, or 0.2% arabinose and 100 μM IPTG. Plates were then incubated at 37°C for 24 hours. Positive interactions yielded single colonies on M9L with 0.2% arabinose and 100 μM IPTG after 24 hours of growth. Intermediate interactions yielded modest growth on plates but no visible single colonies. No intermediate growth phenotypes were observed for the 20x20 matrix (Fig. 2B).

ParD3-ParE3 expression and purification

Recombinant *Mesorhizobium opportunistum* ParDE3 protein complex was expressed in *E. coli* Rosetta(DE3)pLysS (Novagen). A 50 mL overnight culture in LB medium supplemented with 50 μg/ml kanamycin (LB-Kan_{50}) was used to inoculate 2 L of LB-Kan_{50}; this culture was incubated at 37°C in a rotary shaker at 220 rpm. Transcription of recombinant *parDE3*

was induced at an OD_{660} of 0.8 by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 h of induction, the cells were harvested by centrifugation at 12,000g for 20 min at 4°C. Cell pellets were resuspended in 30 ml of lysing/binding buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 10 mM imidazole with 5 µg/ml of DNase I (Sigma-Aldrich) and half a tablet of cOmplet EDTA free protease inhibitor cocktail (Roche Life Science).

Cells were disrupted by one passage through an LV1 microfluidizer (Microfluidics, Westwood, MA) and the cell debris was removed by centrifugation for 20 min at 25,000 *g*. The supernatant was loaded onto a Ni^{2+} Sepharose affinity column (GE Life Sciences) pre-equilibrated with the binding buffer. Two washing steps were performed using 10 mM and 75 mM of imidazole followed by two elution steps with 200 mM and 1 M imidazole in the binding buffer. After purity of the different fractions was assessed by SDS-PAGE, the protein solution was dialyzed against 10 mM Tris (pH 7.4), 150 mM NaCl, 200 mM imidazole buffer.

Crystallization of ParD3-ParE3

Purified ParDE3 was purified and concentrated using a centrifugal filter (3 kDa MWCO, Amicon-Millipore). Protein purity was estimated to be 95% as assessed by 14% SDS-PAGE stained with Coomassie brilliant blue. Initial crystallization screening was carried out using the sitting-drop, vapor-diffusion technique in 96-well microplates (Nunc). Trays were set up using a Mosquito robot (TTP LabTech) and commercial crystallization kits (Nextal-Qiagen). The drops were set up by mixing equal volumes (0.1 µl) of the protein and the precipitant solutions equilibrated against 75 µl of the precipitant solution. In all trials, the protein concentration was ~ 40 mg/mL. In approximately five days, needle-like crystals

appeared in condition 15 of the Pro-complex Suite crystallization kit (Qiagen). After manual refinement of the crystallization condition, the best crystals were obtained at 19°C with the following crystallization solution: 400 mM Sodium Acetate, 100 mM Sodium Citrate pH5.5, 20% PEG 4000, 20 % glycerol. All manual crystallization attempts were carried out using the hanging-drop, vapor-diffusion technique in 24-well plates (Hampton). Prior to flash freezing in liquid nitrogen, drops containing the crystals were mixed with 1 µl of a crystallization solution containing 100 mM sodium iodide and incubated for 4 hours. Crystals were then cryo-protected by soaking them in the crystallization solution containing 25% glycerol and 100 mM sodium iodide.

Crystallographic data collection and data processing

Crystal diffraction was measured at a temperature of 100 K using a 1 degree oscillation range on beamline 21-ID-D (LS-CAT, Advanced Photon Source, Argonne, Illinois); diffraction images were collected on a MAR Mosaic 300 detector. Diffraction images were processed using the Xia[2]. Geometric refinement and examination of the scaled amplitudes revealed that the ParDE3 crystals belong to orthorhombic space group I222, with cell dimensions a=43.18, b=118.84, c=211.42 (α=β=γ=90˚) (see Table S1).

Diffraction from a single ParDE3 protein crystal was measured to 1.53 Å at an energy of 12.66 keV (0.979 Å). The anomalous signal in the data was used to locate iodide atoms in the lattice, and the structure was phased by single wavelength anomalous dispersion (Dauter, 2002)using the Autosol SAD routine in Phenix (Adams et al., 2010). Two ParDE3 complexes are present in the asymmetric unit. Eight iodine sites were located within the asymmetric unit. A preliminary ParDE3 structural model was built *de novo* from the initial

experimental, solvent-flattened maps using the AutoBuild routine and phenix.refine. This initial model was then manually examined and corrected; solvent addition and refinement of the structure was conducted iteratively using Coot (Emsley and Cowtan, 2004) and phenix.refine (Adams et al., 2010). The final structural model was refined to an R_{work} of 16.85% and R_{free} of 19.54%. Coordinates of ParDE3 were deposited in the Protein Data Bank (PDB ID 5CEG). Crystallographic data and refined model statistics are in Table S1.

Size exclusion chromatography

A purified sample of ParDE3 (10 mg/ml-300 µl) was injected on a GE Healthcare Superdex 200 10/300 GL column (flow rate 0.5 ml/min) and fractions of 500 µl were collected. 10 mM Tris pH 7.4, 150 mM NaCl, 200 mM imidazole was used as a running buffer. Collected fractions were resolved on 14% SDS-PAGE gels and compared to the elution profile. To estimate the molecular weight and, hence, oligomeric state of the ParDE3 complex in solution, its elution volume was compared to molecular weight standards (blue dextan, aldolase, conalbumin and ovalbumin) resolved on the same column using the same buffer and flow protocol.

ParD3 library construction

Residues incorporated at each library position were chosen to closely resemble that of naturally occurring ParD homologs. Briefly, the software HMMER was used to identify and align homologs of *C. crescentus* ParD3 using an E-value cutoff of 0.0001, and then sequences greater than 95% identical were removed. For each library position, amino acid frequencies were extracted from the curated ParD3 alignment, and a library residue set was chosen that covered at least 95% of the sequence diversity in the ParD3 alignment.

This approach yielded a total of 12, 6, 13, and 10 residues at the four positions of the library.

The ParD3 library was generated using the ProxiMAX technique (Ashraf et al., 2013). The main advantage of this technique over traditional NNS libraries is that the amino acid composition at each position in the library can be specified, thus reducing the complexity of the library. To generate the ParD3 library, we began with an "acceptor" fragment that contained the region of *parD3* upstream of the first position in the library (L59) and different hairpin "donor" fragments that contain (i) one of the codons we wish the incorporate and (ii) a downstream *MlyI* restriction site. We set up separate blunt-end ligations between the acceptor fragment (0.1 μM) and each codon donor fragment (0.5 μM) using T4 ligase and incubated at 22°C for 1 hour. These ligations were diluted 1:100 in water and used as a template for separate high-fidelity PCR (Phusion) using primers specific for the acceptor and donor fragments. These PCR reactions were gel purified, quantified, and then pooled in equimolar amounts. The pooled mixture was then cut using MlyI and PCR purified, resulting in a new acceptor fragment that contains the L59 position randomized. This acceptor fragment was then used for three additional rounds of ProxiMAX randomization as outlined above, to create a library in positions L59, W60, D61, and K64 in ParD3. The final fragment was sub-cloned into the SacI and PvuI sites of pEXT20 and library composition was verified by Illumina sequencing of the relevant region of *parD3*.

Illumina-based sequencing and fitness calculations

Plasmid DNA was extracted from frozen cell samples (Qiagen) and used as a template for PCR reactions (20 cycles) with custom barcoded primers containing Illumina flowcell adaptor sequences. The samples were multiplexed and run on an Illumina HiSeq instrument. Multiplexed Illumina reads from a single lane were sorted based on an exact match to a four-letter barcode sequence. Reads were then filtered to remove sequences that (a) contained frameshift mutations, (b) encoded for a *parD3* variant not in the planned library, or (c) lacked an exact match to six nucleotides before (AGGCAG) and after (GCAAGC) the randomized region. Sequences that passed these quality filters were then counted and frequency-normalized. We calculated the fitness of each variant as described previously (van Opijnen et al., 2009). Briefly, we generated a linear fit to the frequencies of each mutant as a function of time, and then calculated the log-fold expansion of each mutant relative to the rest of the population, yielding W_{raw} for each variant:

$$W_i = \frac{log(E(\frac{t_1}{t_0}))}{log(E\frac{(1-t_1)}{(1-t_0)})}$$

where t_0 is the frequency of the mutant at 200 min, t_1 is the frequency of the mutant at 600 min, and E is the expansion factor of the culture (OD at t_0 / OD at t_1).We then transformed these raw fitness values such that the *W* value for frameshift variants was 0 and the *W* value for the wild-type (LWDK) sequence was 1.

Creation of the orthogonal ParE3* toxin

To create a ParE3 toxin with a novel specificity profile, we focused on residues in ParE3 that covary with W60/D61 from ParD3. We found that residues R58/A61/L72 in ParE3 covary with W60/D61 from ParD3 with a GREMLIN scaled score greater than 1. We then searched for residues within ParE3 that covary with R58/A61/L72 (termed "supporting residues") with a GREMLIN scaled score greater than 1. Repeating this search process iteratively produced two more supporting residues in ParE3, M63/R54, for a total of five specificity and supporting residues in ParE3: R54/R58/A61/M63/L72.

To identify which mutations to make in these five residues, we searched naturally existing ParE sequences for combinations of residues that often occur at these positions. We chose to incorporate the residue combination VEIRF because each residue was commonly observed at the corresponding position in naturally existing ParE sequences and because it was chemically dissimilar to the wild-type residue at that position, RRAML.

Generation of force-directed graphs

Graphs were generated using networkx and visualized using Gephi (Jacomy et al., 2014). For the graph in Figure 5C, edges were drawn for every two ParD3 variants that are separated by a single amino acid substitution; for the graph in Figure S5B, edges were drawn for every two ParD3 variants that are separated by single nucleotide substitutions. Nodes were colored based on specificity class using custom-written Python scripts. The layout was generated using the Force Atlas algorithm to completion.

Table S1. Data collection and refinement statistics; Related to Figure 3.

Wavelength (Å)	0.9785
Resolution range (Å)	31.3-1.59
Space group	I 2 2 2
Unit cell	43.18, 118.84, 211.42, 90 90 90
Total reflections	664000
Unique reflections	73683
Completeness (%)	99.95
Mean I/sigma(I)	13.46
Wilson B-factor	16.30
R-merge	0.1044
Reflections used for R-free	3809
R-work	0.1685
R-free	0.1954
RMS(bonds)	0.007
RMS(angles)	1.07
Ramachandran favored (%)	98.4
Ramachandran outliers (%)	0
Clashscore	3.09
Average B-factor	22.30

Statistics for the highest-resolution shell are shown in parentheses.

Supplemental References

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