SUPPLEMENTAL INFORMATION

Branched signal wiring of an essential bacterial cell-cycle phosphotransfer protein

Jimmy A. Blair#[1,5], Qingping Xu#[2,3], W. Seth Childers#[1], Irimpan I. Mathews[2], Justin W. Kern[1], Michael Eckart[4], Ashley M. Deacon[2,3,*], and Lucy Shapiro[1,*]

[1]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA, 94305
[2]Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, Menlo Park, CA, 94025
[3]Joint Center for Structural Genomics
[4]Stanford Protein and Nucleic Acid Facility, Stanford University School of Medicine, Beckman Center, Stanford, CA 94305
[5]Present address: Department of Chemistry, Williams College, Williamstown, MA 01267

[#]Authors contributed equally to this work.

[*]Correspondence: adeacon@slac.stanford.edu (A.M.D.); shapiro@stanford.edu, Tel. (+1) 650 725 7678, Fax (+1) 650 725 7739 (L.S.)

Inventory of Supplemental Information:

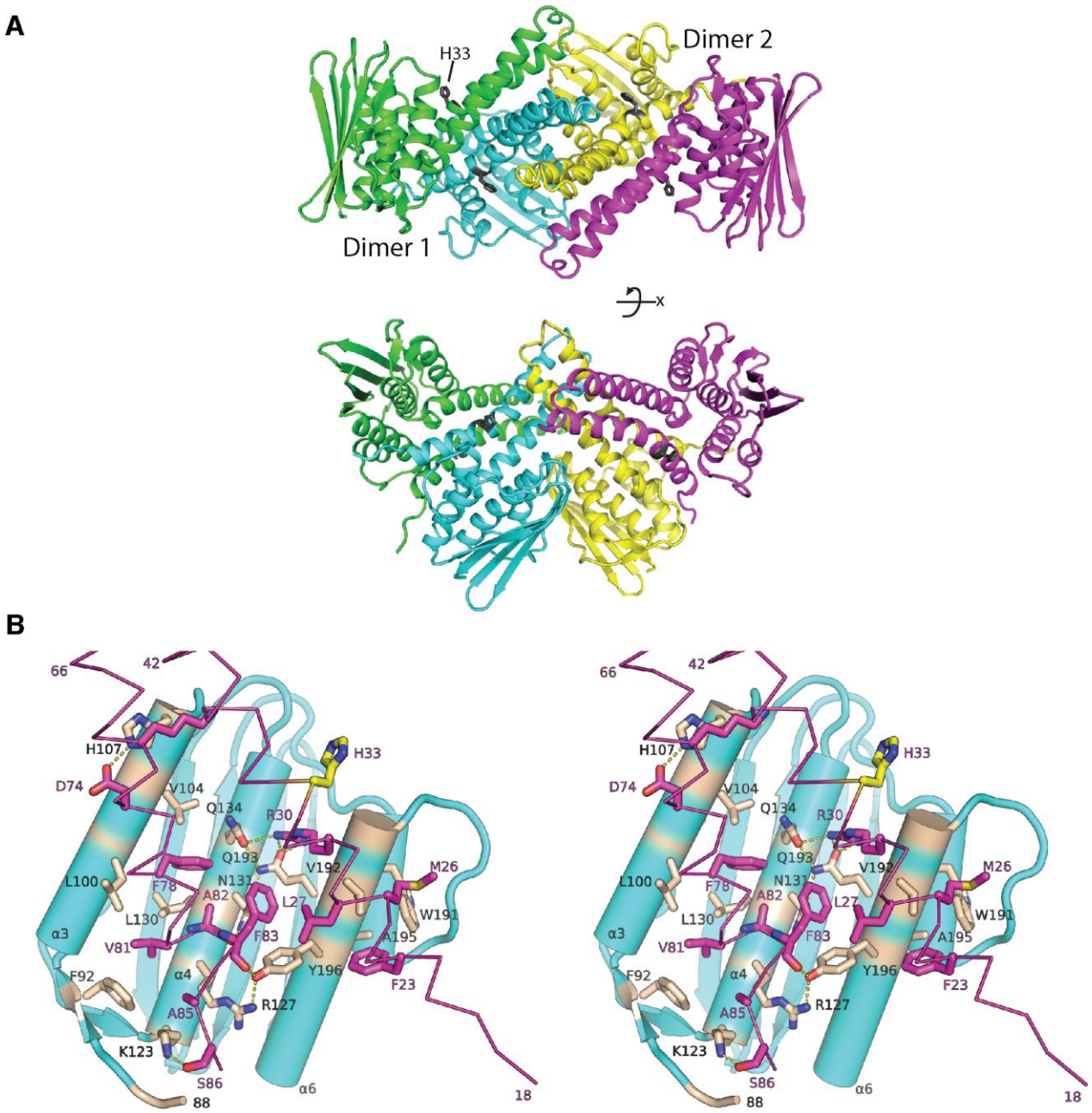


FIGURE S1. (A) The two ChpT dimers in the asymmetric unit. Each chain is shown in a different color, and the phosphorylatable His33 is shown as gray sticks. (B) Stereoview of the ChpT. Inter-helical interactions dominate the DHp-CA domain interface (DHp, magenta and CA, cyan).

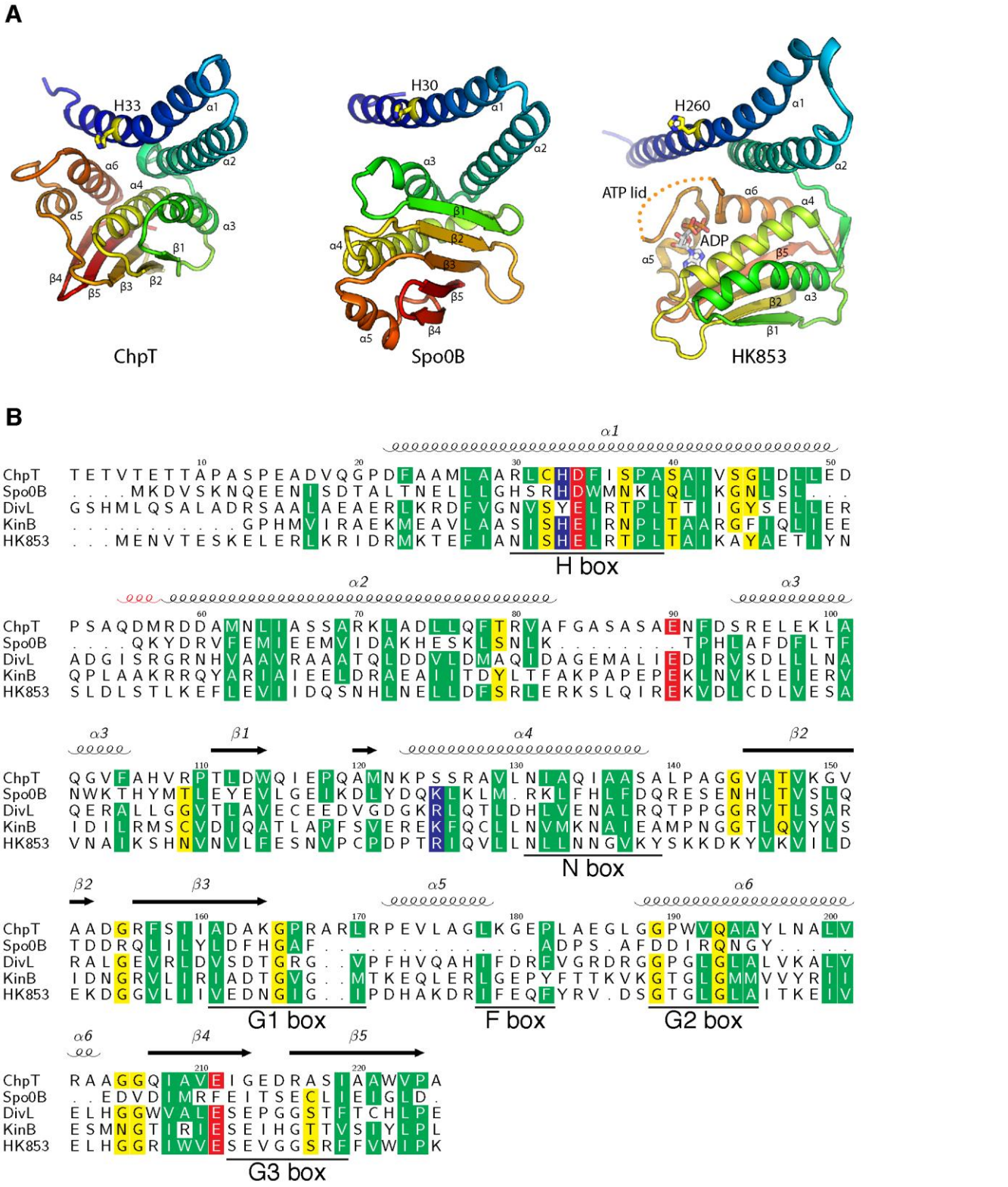
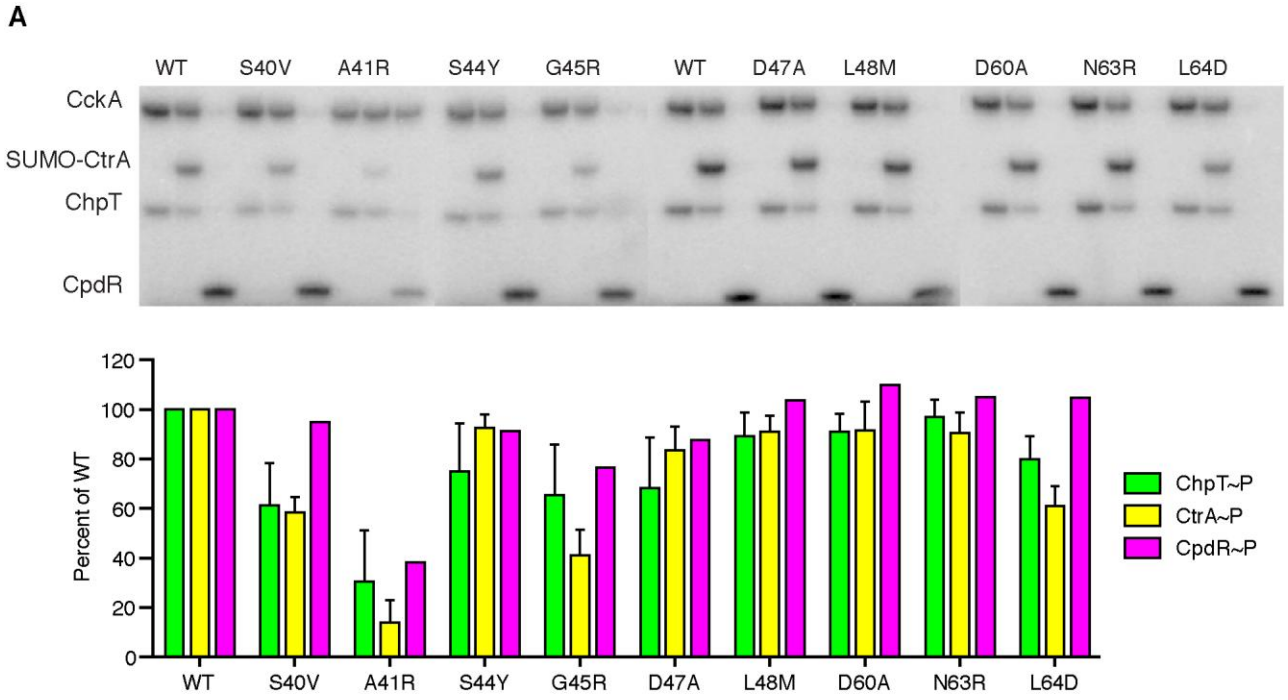


FIGURE S2. A comparison of structurally related phosphosignaling proteins. A. ChpT, Spo0B (PDB ID 1ixm), and HK853 (PDB ID 2c2a) are shown in similar orientations. Each structure is colored in a color-gradient ranging from blue (N-term) to red (C-term). The phosphorylatable histidine and ADP in HK853 are drawn as sticks. B. Sequence alignment of HPts (ChpT and Spo0B), a pseudo-HK (DivL, which does not have a phosphorylatable histidine), and HKs (KinB and HK853). The locations for the HK sequence motifs (H, N, G1, F, G2, and G3 boxes) are marked at the bottom, while the secondary structures of ChpT are shown at the top. Conserved residues

are highlighted in colors (red, acidic; blue, basic; yellow, polar uncharged and green, hydrophobic nonpolar).



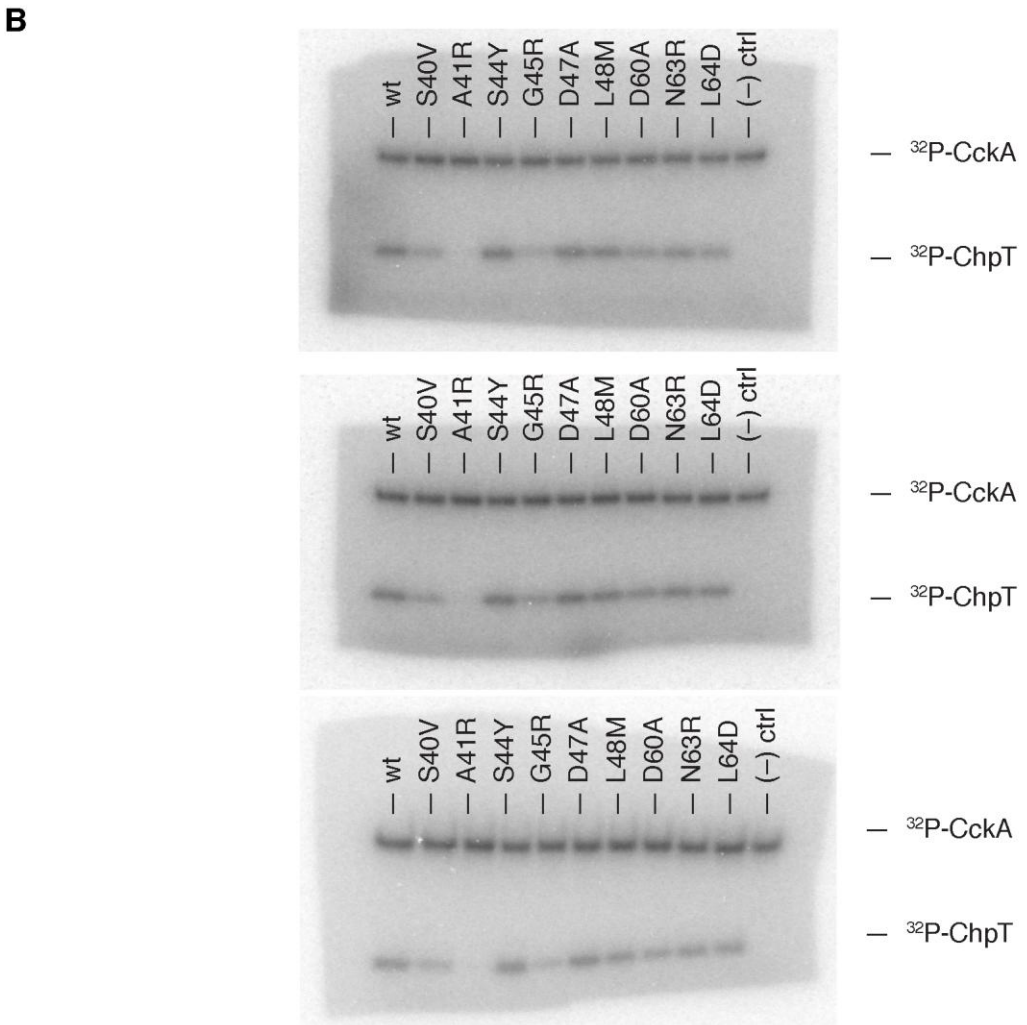
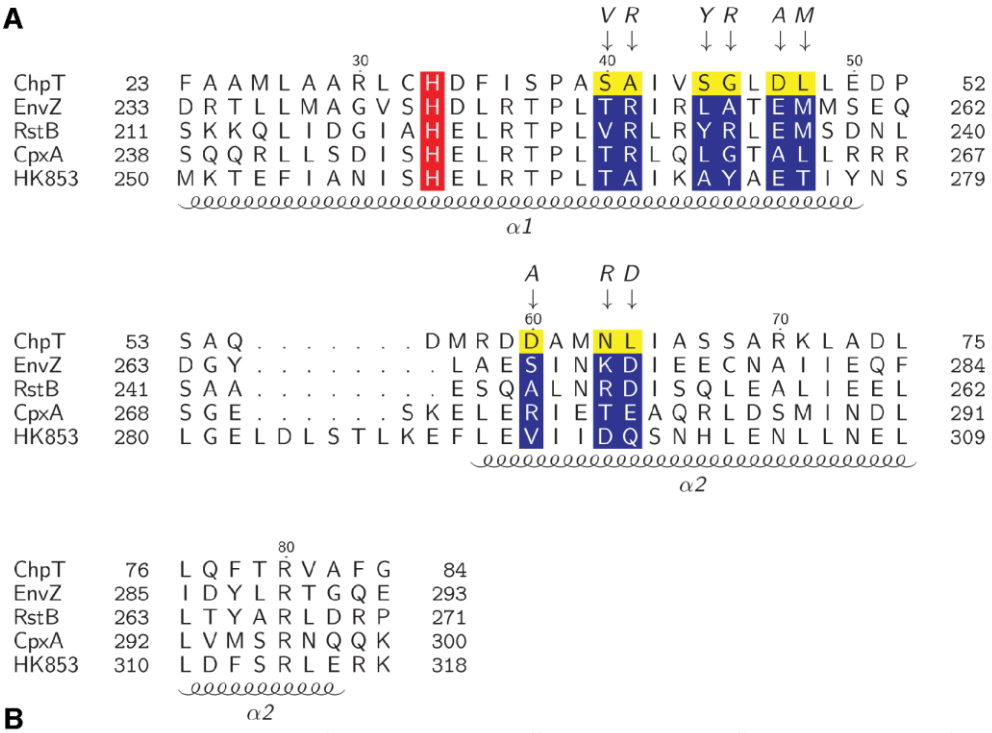


FIGURE S3. (A) Overall phosphorelay output for each mutant. Upper panel: three reaction mixtures with [γ-^{32}P]

ATP for 30 minutes were analyzed: (1) CckA, ChpT, (2) CckA, ChpT, and SUMO-CtrA, and (3) CckA, ChpT, and CpdR. The phosphoproteins were separated by SDS-PAGE and imaged by phosphor storage. Lower panel: quantitation of three replicate phosphotransfer assays (upper panel) and standard deviation for each band. (B) Phosphotransfer assay gels of CckA and ChpT in triplicate. CckA~P autophosphorylated with [γ-^{32}P] ATP was mixed with each ChpT variant and allowed to react for 10s before being stopped. The phosphoproteins were separated by PAGE and imaged by phosphor storage. The negative control lane was a reaction mixture containing [γ-^{32}P] autophosphorylated CckA~P without added ChpT. Shown are the entire gels for three individual experiments.

A


V R
Y R
A M
ChpT 23 FAAMLAARLCHDFISPASAIVSGLDLLEDP 52
EnvZ 233 DRTLLMAGVSHDLRTPLTRIRLATEMMSEQ 262
RstB 211 SKKQLIDGIAHELRTPLVRLRYRLEMSDNL 240
CpxA 238 SQQRLLSDISHELRTPLTRLQLGTALLRRR 267
HK853 250 MKTEFIANISHELRTPLTAIKAYAETIYNS 279
α1
A
R D
ChpT 53 SAQ.....DMRDDAMNLIASSARKLADL 75
EnvZ 263 DGY.....LAESINKDIEECNAIIEQF 284
RstB 241 SAA.....ESQALNRDISQLEALIEEL 262
CpxA 268 SGE.....SKELERIETEAQRLDSMINDL 291
HK853 280 LGELDLSTLKEFLEVIIDQSNHLENLLNEL 309
α2
ChpT 76 LQFTRVAFG 84
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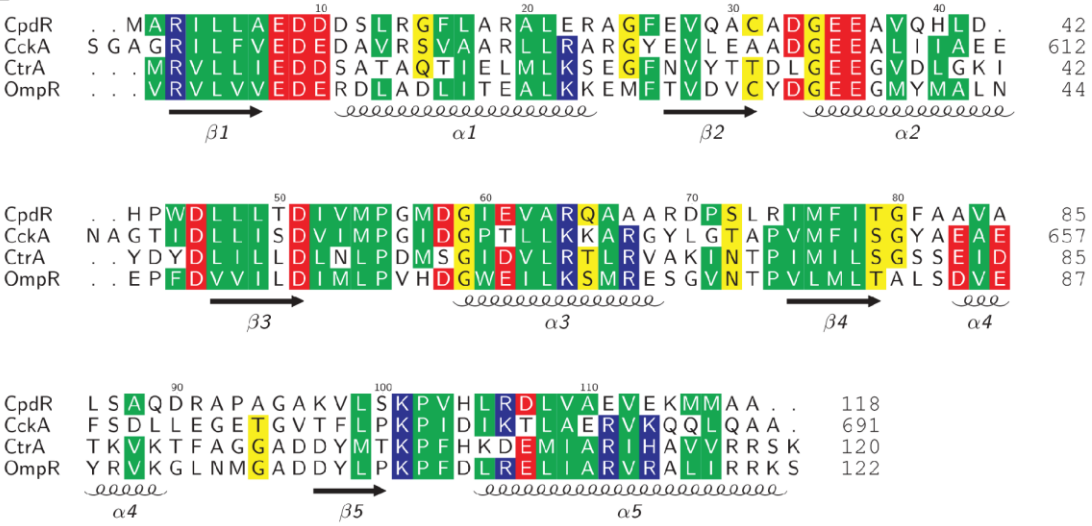
RstB 263 LTYARLDRP 271
CpxA 292 LVMSRNQQK 300
HK853 310 LDFSRLERK 318
α2

B


CpdR ..MARILLAEDDDSLRGFLARALERAGFEVQACADGEEAVQHLD. 42
CckA SGAGRILFVEDEDAVRSVAARLLRARGYEVLEAADGEEALIIAEE 612
CtrA ...MRVLLIEDDSATAQTIELMLKSEGFNVYTTDLGEEGVDLGKI 42
OmpR ...VRVLVVEDERDLADLITEALKKEMFTVDVCYDGEEGMYMALN 44
β1
α1
β2
α2
CpdR ..HPWDLLLTDIVMPGMDGIEVARQAAARDPSLRIMFITGFAAVA 85
CckA NAGTIDLLISDVIMPGIDGPTLLKKARGYLGTAPVMFISGYAEAE 657
CtrA ..YDYDLILLDLNLPDMSGIDVLRTLRVAKINTPIMILSGSSEID 85
OmpR ..EPFDVVILDIMLPVHDGWEILKSMRESGVNTPVLMLTALSDVE 87
β3
α3
β4
α4
CpdR LSAQDRAPAGAKVLSKPVHLRDLVAEVEKMMAA.. 118
CckA FSDLLEGETGVTFLPKPIDIKTLAERVKQQLQAA. 691
CtrA TKVKTFAGGADDYMTKPFHKDEMIARIHAVVRRSK 120
OmpR YRVKGLNMGADDYLPKPFDLRELIARVRALIRRKS 122
α4
β5
α5

C

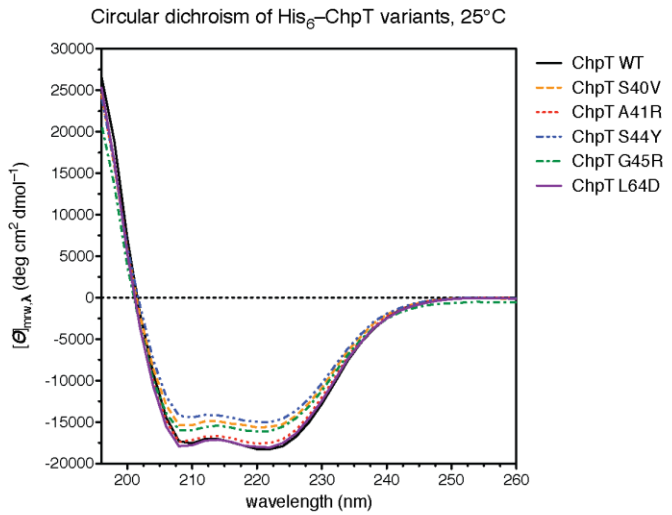

Circular dichroism of His6–ChpT variants, 25°C
[Θ]mrw,λ (deg cm2 dmol−1)
wavelength (nm)
ChpT WT
ChpT S40V
ChpT A41R
ChpT S44Y
ChpT G45R
ChpT L64D

FIGURE S4. (A) A structure-based sequence alignment of the DHp domains of ChpT and several HKs guide the design of point mutations along the DHp-RR binding interface. Highlighted in red is the phosphorylatable histidine residue. Highlighted in blue are the residues identified by Skerker *et al*. (Skerker et al., 2008) among EnvZ, RstB, CpxA, and HK853 as responsible for maintaining selectivity for cognate RRs. Highlighted in yellow are the putative ChpT residues believed to control selectivity for its RRs. Residues above arrows are the point mutations generated at that that site for ChpT. (B) Sequence alignment of the RDs of the ChpT cognate signaling partners with OmpR. The secondary structures of OmpR (PDB ID 1kgs) are shown below the sequences. Conserved residues are highlighted in colors (red, acidic; blue, basic; yellow, polar uncharged and green, hydrophobic nonpolar). (C) Wavelength scan circular dichroism of six ChpT variants reveals that each protein is well folded and displays similar secondary structure features.

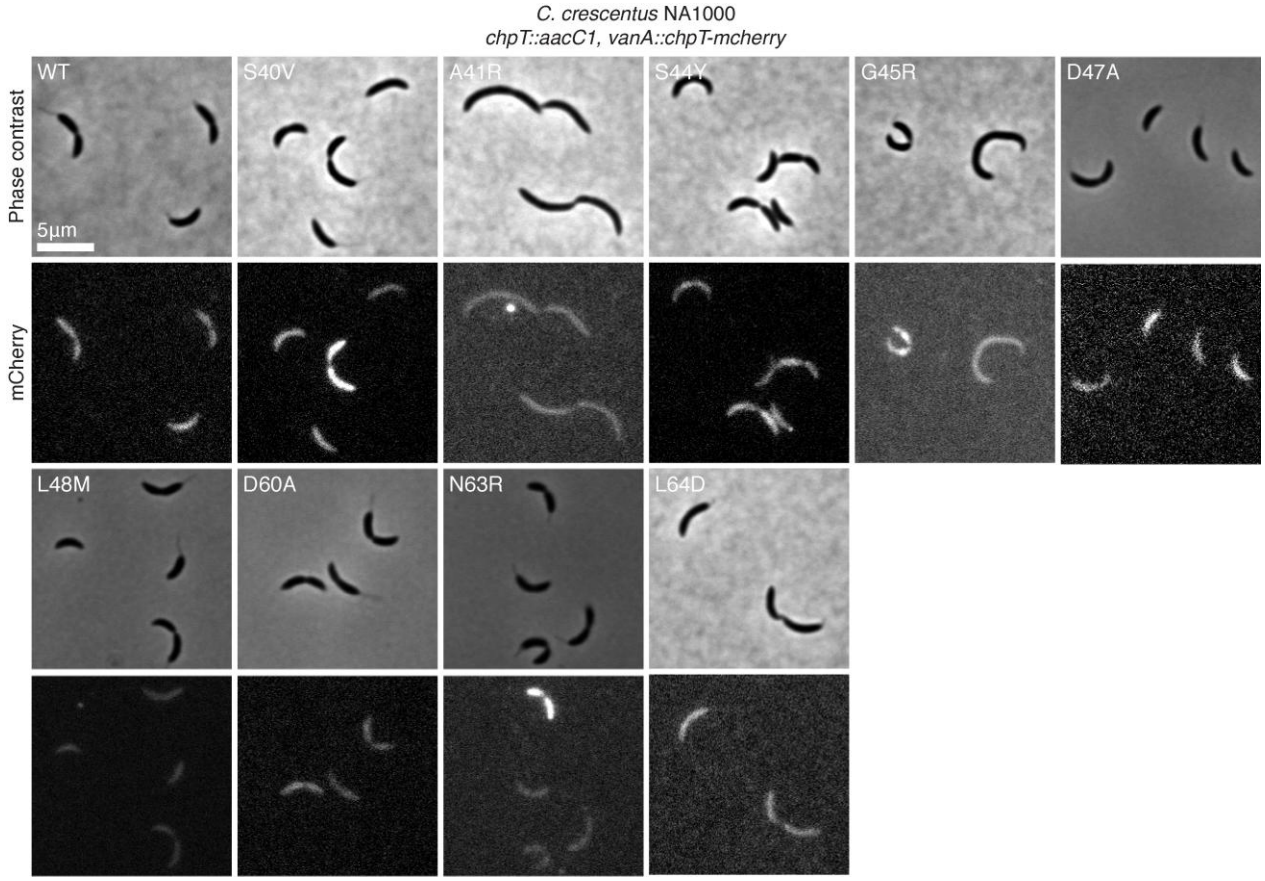


FIGURE S5. ChpT is diffuse throughout the cell. Fluorescence light microscopy images of *C. crescentus* NA1000 strains whose sole ChpT copy is either wild-type ChpT or a ChpT variant harboring a point mutation in the RR binding region of ChpT. Phase contrast and mCherry-fluorescence images are shown.

SUPPLEMENTAL STRUCTURAL ANALYSIS

Domain interface between CA and DHp—The inter-domain arrangement between the CA domain and the DHp domain differs significantly between ChpT and related proteins such as Spo0B and HK853 (Fig. S2). The interface between the CA and DHp domains in ChpT is mediated by helical interactions (Fig. S1B) and buries a total surface area of ~900 $Å^2$. The interactions are mainly hydrophobic involving the N-terminal section of α1 (Phe23, Met26, Leu27) and the C-terminal section of α2 (Leu75, Phe78, Val81, Ala82, Phe83 and Ala85) from DHp, and helices α3, α4, and α6 from CA. Hydrogen bonds also contribute significantly to the domain interface (residues Arg30, Gln134, Gln193, Asn131, Asp74, His107, Tyr196, Arg127, Ser86 and Lys123). Most of the residues involved in the domain interface are highly conserved among ChpT orthologs (Fig. 2B).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all chemicals were purchased from Sigma Aldrich and used directly without further purification. Restriction and DNA-modifying enzymes were purchased from New England Biolabs or Fermentas. PCR reactions were performed in 50 μL reaction mixtures containing 5% (v/v) DMSO, 0.2 μM each primer, and 0.2 mM each dNTP, and either KOD polymerase (Novagen) or Phusion HS II DNA Polymerase (Thermo Scientific) was used. Site-directed mutagenesis was performed using the protocols described in the QuikChange mutagenesis kit (Agilent Technologies) with the following changes: Pfu Ultra polymerase

(Agilent Technologies) was used in 50 µL reaction mixtures containing 5% (v/v) DMSO, 0.2 µM each primer, and 0.2 mM each dNTP, and a 55°C annealing temperature was used. Oligonucleotides were synthesized by the Stanford PAN Facility, and all DNA sequencing reactions were performed by Sequetech (Mountain View, CA). Antibiotic concentrations used in liquid growth selection of *E. coli*: kanamycin sulfate (30 µg/mL) and chloramphenicol (20 µg/mL). Antibiotic concentrations used in liquid growth selection of *Caulobacter crescentus*: kanamycin sulfate (5 µg/mL) and gentamycin (1 µg/mL). Radiolabeling experiments with [γ-^{32}P] ATP were conducted with 6000Ci/mmol 10mCi/ml EasyTides solution (Perkin Elmer). The plasmids, strains, and oligonucleotides used in this study are listed in Tables S2, S3, and S4, respectively.

ChpT Cloning, Expression, and Purification—A fragment of genomic sequence containing *chpT* was amplified from *Caulobacter crescentus* genomic DNA (gift from M. Schwartz) using primers JABp69 and JABp68 and cloned at the NdeI and SacI restriction sites into the bacterial expression vector pET-28b(+) (Novagen), which encodes an N-terminal His_6-tag and a thrombin cleavage site. These steps produced plasmid pJAB29, which contains the full, misannotated *chpT* gene as reported in the Caulobacter crescentus CB15 genome (Nierman et al., 2001). Chen *et al.* demonstrated that ChpT protein produced in *Caulobacter crescentus* starts at Methionine-29, as annotated in the CB15 genome (Chen et al., 2010); these results were independently confirmed by Christen and co-workers (Christen et al., 2011). To produce ChpT of the correct length, *chpT* was subcloned from pJAB29 using primers JABp72 and JABp69 into pET-28b(+) at the NdeI and SacI restriction sites to yield plasmid pJAB30. Sequence verified pJAB30 was transformed into the Rosetta(DE3) pLysS *E. coli* expression strain (Novagen) to produce strain JAB75. A single colony of JAB75 was used to inoculate 50 mL of LB broth supplemented with kanamycin sulfate (30 µg/mL) and chloramphenicol (20 µg/mL) that was grown overnight at 37°C in a shaking incubator. The following morning, 20 mL of the overnight culture was used to inoculate 2 L of LB broth supplemented with kanamycin sulfate (30 µg/mL) and chloramphenicol (20 µg/mL) and grown at 37°C in a shaking incubator to an optical density of 0.6, as measured by absorbance at 600 nm. The culture was transferred to 25°C, and ChpT expression was induced by addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) to 333 µM. After four hours, the cells were harvested by centrifugation (3700 × g, 4°C, 20 min), resuspended in 4°C PBS pH 7.4, harvested by centrifugation (3700 × g, 4°C, 20 min), and the wet cell pellet stored at –80°C until use. Frozen cell pellets were thawed on ice and resuspended in 25 mL lysis buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM DTT, 250U Benzonase nuclease (Sigma), SigmaFast protease inhibitor cocktail (Sigma)). Cells were lysed using three passes through a French Press (20000 psi, 4°C), and the insoluble cell debris was removed by centrifugation (29500 × g, 4°C, 30 min). His_6-ChpT protein was affinity purified in batch: 3 mL 50% slurry of Ni-NTA agarose affinity resin (Qiagen) equilibrated in lysis buffer was incubated with the soluble, cleared lysate at 4°C for 60 min, the slurry was applied to a chromatography column, the resin bed was washed with 120 mL Ni-NTA wash buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM DTT), and the protein was eluted with 10 mL Ni-NTA elution buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 250 mM imidazole, 10% (v/v) glycerol, 1 mM DTT) directly into an Amicon-Ultra 15mL 10k MWCO concentrator. The eluted protein solution was concentrated to 2.5 mL by centrifuge ultrafiltration (3700 × g, 4°C, 30 min) and buffer exchanged into CleanCleave buffer (50 mM Tris·HCl pH 8.0, 100 mM NaCl, 10 mM $CaCl_2$, 10% (v/v) glycerol) using a PD-10 desalting column (GE Healthcare) that yields a final eluted volume of 3.5 mL. Removal of the His_6-tag was achieved by incubating the protein solution with 1 mL of 50% slurry of solid-supported thrombin (CleanCleave thrombin kit, Sigma) equilibrated in CleanCleave buffer at 4°C overnight on a rocking platform, applying the slurry to a chromatography column the next morning and collecting the eluate. Undigested His_6-ChpT was removed by subtractive Ni-NTA agarose affinity purification: 1 mL 50% slurry Ni-NTA agarose (Qiagen) equilibrated in CleanCleave buffer was incubated with the protein solution for 1 h, the slurry was applied to a chromatography column, and the eluate was collected. The eluate was concentrated to 1.5 mL by centrifuge ultrafiltration (3700 × g, 4°C) using an Amicon Ultra 15 10 k MWCO, and purified by size exclusion chromatography on a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) equilibrated in kinase storage buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 200 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT) to yield approximately 33 mg of purified ChpT from 2 L of bacterial culture. Removal of the His_6-tag was confirmed by western blot using the HisProbe (Pierce) affinity reagent, which specifically binds to the His_6-tag. The protein was concentrated by centrifuge ultrafiltration (3700 × g, 4°C) using an Amicon Ultra 15 10 k MWCO to 13 mg/mL, flash frozen in liquid nitrogen and stored at –80°C. The protein concentration was determined by

measuring absorbance at 280 nm and using the calculated molar extinction coefficient of 18115 cm^{-1} M^{-1}. The calculated molecular weight of this construct is 23649 Da.

His_6-ChpT variants containing point mutations in the putative RD binding region of the ChpT DHp domain were generated by Quikchange mutagenesis of pJAB30 using protocols described above. Primers used are as follows: S40V (JABp115, JABp116); A41R (JABp117, JABp118); S44Y (JABp119, JABp120); G45R (JABp121, JABp122); D47A (JABp123, JABp124); L48M (JABp125, JABp126); D60A (JABp127, JABp128); N63R (JABp129, JABp130); L64D (JABp131, JABp132). Purified plasmids were sequence-verified, transformed in to Rosetta(DE3) pLysS *E. coli* (strains JAB134-JAB139, JAB143-JAB145), and expressed and purified as described above except for the following changes: following the initial Ni-NTA affinity purification, the proteins were exchanged in to kinase storage buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 200 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT) using PD-10 desalting columns (GE Healthcare), concentrated by centrifuge ultrafiltration (3700 × g, 4°C) using an Amicon Ultra 15 10 k MWCO to approximately 10 mg/mL, flash frozen in liquid nitrogen, and stored at –80°C.

CckA Cloning, Expression, and Purification—The cytoplasmic region of CckA, $CckA_{70\text{-}691}$, was subcloned from pNJH87 using primers JABp64 and JABp66 into pET-28b(+) (Novagen) at NdeI and SacI restriction sites. These steps produced plasmid pJAB27, which was sequence verified and used to transform chemically competent Rosetta(DE3) pLysS *E. coli* cells (Novagen) to produce strain JAB70. A single colony of JAB70 was used to inoculate 50 mL of LB broth supplemented with kanamycin sulfate (30 µg/mL) and chloramphenicol (20 µg/mL) that was grown overnight at 37°C in a shaking incubator. The following morning, 20 mL of the overnight culture was used to inoculate 2 L of LB broth supplemented with kanamycin sulfate (30 µg/mL) and chloramphenicol (20 µg/mL) and grown at 37°C in a shaking incubator to an optical density of 0.6, as measured by absorbance at 600 nm. The culture was transferred to 25°C, and $CckA_{70\text{-}691}$ expression was induced by addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) to 333 µM. After five hours, the cells were harvested by centrifugation (3700 × *g*, 4°C, 20 min), resuspended in 4°C PBS pH 7.4, harvested by centrifugation (3700 × *g*, 4°C, 20 min), and stored at –80°C until use. Frozen cell pellets were thawed on ice and resuspended in 25 mL lysis buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM DTT, 250U Benzonase nuclease (Sigma), SigmaFast protease inhibitor cocktail (Sigma)). Cells were lysed using four passes through a French Press (20000 psi, 4°C), and the insoluble cell debris was removed by centrifugation (29500 × *g*, 4°C, 30 min). His_6-$CckA_{70\text{-}691}$ protein was affinity purified in batch: 4 mL 50% slurry of Ni-NTA agarose affinity resin (Qiagen) equilibrated in lysis buffer was incubated with the soluble, cleared lysate at 4°C for 60 min, the slurry was applied to a chromatography column, the resin bed was washed with 120 mL Ni-NTA wash buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM DTT) and the protein was eluted with 10 mL Ni-NTA elution buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 250 mM imidazole, 10% (v/v) glycerol, 1 mM DTT). The eluted protein solution was buffer exchanged into QA buffer (50 mM Tris·HCl pH 8.0, 50 mM KCl 10% (v/v) glycerol) using four PD-10 desalting columns (GE Healthcare). The protein solution was purified by anion exchange chromatography: the sample was applied to a 1 mL HiTrap Q Sepharose FF column equilibrated in QA buffer using a GP-250 FPLC gradient programmer (Pharmacia, now GE Healthcare) equipped with dual P-500 pumps (Pharmacia, now GE Healthcare), the column was washed with 10 column volumes of QA buffer, the protein was eluted with a linear gradient of QB buffer (50 mM Tris·HCl pH 8.0, 1 M KCl 10% (v/v) glycerol) from 0 to 100% QB, and peak fractions containing His_6-$CckA_{70\text{-}691}$ were pooled. The sample was concentrated to 1.5 mL by centrifuge ultrafiltration (3700 × *g*, 4°C) with an Amicon Ultra 4 30k MWCO (Millipore) and purified by size exclusion chromatography on a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) equilibrated in kinase storage buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 200 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT) using a GP-250 FPLC gradient programmer (Pharmacia, now GE Healthcare) equipped with dual P-500 pumps (Pharmacia, now GE Healthcare) to yield approximately 6.8 mg of purified His_6-$CckA_{70\text{-}691}$ from 2 L of bacterial culture. The protein was concentrated by centrifuge ultrafiltration (3700 × *g*, 4°C) using an Amicon Ultra 15 10 k MWCO to 4.5 mg/mL, flash frozen in liquid nitrogen and stored at –80°C. Protein purity was assessed to be >95% pure by Coomassie-stained polyacrylamide gel electrophoreses. The protein concentration was determined by measuring absorbance at 280 nm and using the calculated molar extinction coefficient of 28880 cm^{-1} M^{-1}. The calculated molecular weight of this construct is 68505 Da.

A variant of CckA unable to undergo autophosphorylation, His_6-$CckA_{70\text{-}691}$-H322A, was generated using the

megaprimer method as described by Ke and Madison (Ke and Madison, 1997). The initial round of amplification used primers JABp85 and JABp88 in a reaction containing the following components: 26.5 µL nuclease free water, 10 µL 5× QC buffer, 5 µL mixture of 2 mM each dNTPs (EMD Millipore), 5 µL pJAB27 diluted 1:20 with EB from a 5 mL culture miniprep, 1.5 µL 100 µM JABp85, 1.5 µL 10 µM JABp88, 2.5 µL DMSO and 0.5 µL Phusion HS II DNA Polymerase (Thermo Scientific). The PCR was initiated by melting at 98°C for 3 min, followed by 25 cycles of melting at 98°C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30s, followed by extension at 72 °C for 5 min and stored at 4°C overnight. To the resulting PCR product was added 1.5 µL 100 µM JABp86, 2.5 µL mixture of 2 mM each dNTPs (EMD Millipore) and 0.5 µL 0.5 µL Phusion HS II DNA Polymerase (Thermo Scientific), and PCR was initiated by melting at 98°C for 1 min, followed by 25 cycles of melting at 98°C for 30 s, annealing and extension at 72°C for 55s, followed by extension at 72 °C for 5 min. The resulting PCR product was cloned into pET-28b(+) (Novagen) at the NcoI and SacI sites. These steps produced plasmid pJAB35, which encodes *cckA*$_{71\text{-}691}$-H322A with a C-terminal His$_6$-tag. The expression plasmid for His$_6$-CckA$_{70\text{-}691}$-H322A, pJAB36, was generated by subcloning *cckA*$_{70\text{-}691}$-H322A from pJAB35 using the protocol described above for generation of pJAB27 with primers JABp64 and JABp66. His$_6$-CckA$_{70\text{-}691}$-H322A was expressed from strain JAB90 (Rosetta(DE3) pLysS *E. coli* transformed with pJAB36) and purified as described above for His$_6$-CckA$_{70\text{-}691}$. A calculated molar extinction coefficient of 28880 cm^{-1} M^{-1} was used for protein concentration measurements at 280 nm, and the calculated molecular weight of this construct is 68439 Da.

The CckA receiver domain, CckA-RD (aa 571–691), was subcloned from pNJH87 using primers JABp65 and JABp66 into pET-28b(+) (Novagen) at NdeI and SacI restriction sites. These steps produced plasmid pJAB28, which was sequence verified and used to transform chemically competent Rosetta(DE3) pLysS *E. coli* cells (Novagen) to produce strain JAB73. CckA-RD was expressed and purified from a 2L culture of strain JAB73 using the same methods described for ChpT purification. Briefly, these steps included Ni-NTA affinity purification, overnight cleavage of the His$_6$-tag with CleanCleave thrombin (Sigma), subtractive Ni-NTA affinity purification, and gel filtration to produce 15 mg CckA-RD. The purified CckA-RD was concentrated to 10 mg/mL using an Amicon Ultra 15 10 k MWCO concentrator, flash frozen in liquid nitrogen and stored at –80°C. A calculated molar extinction coefficient of 4470 cm^{-1} M^{-1} was used for protein concentration measurements at 280 nm, and the calculated molecular weight of this construct is 13430 Da. To generate FLAG-CckA(70–691) the FLAG peptide sequences was embedded within PCR primers WSCp29 and CckA(70–691) was amplified using primers WSCp29 and WSCp30 and pET-28b(+) (Novagen) at NdeI and SacI restriction sites to generate expression vector pWSC19. FLAG-CckA was subsequently expressed and purified as described above.

CpdR Expression and Purification—CpdR was expressed and purified using methods similar to those described by Abel *et al*. (Abel et al., 2011). Strains BPC223 and BPC250 were kind gifts from Peter Chien (UMass Amherst) (Abel et al., 2011).

Plasmid pET28b-*ulp1*, which is used to express the catalytic domain of SUMO protease as a C-terminally-tagged His$_6$ fusion, was purified by miniprep from strain BPC250, an *E. coli* DH5α strain harboring pET28b-*ulp1* (Wang et al., 2007). Plasmid pET28b-*ulp1* was used to transform chemically competent Rosetta(DE3) pLysS *E. coli* cells (Novagen) to produce strain JAB141. SUMO-His$_6$ protease was expressed and purified from a 2L culture of strain JAB141 using similar methods described for ChpT with the following changes: SUMO-His$_6$ protease was purified by Ni-NTA affinity purification; buffer exchanged using two PD-10 desalting columns (GE Healthcare) into 7 mL of 40 mM Tris·HCl pH 8.0, 300 mM NaCl, 2 mM EDTA; protein concentration of 6.7 mg/mL was determined by Branford reagent (Sigma) using BSA as a standard; diluted with 7 mL of 40 mM Tris·HCl pH 8.0, 300 mM NaCl, 2 mM EDTA and 14 mL of glycerol to provide 28 mL of 1.7 mg/mL SUMO-His$_6$ protease in 20 mM Tris·HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 50% (v/v) glycerol, which was stored at –80°C until use.

His$_6$-SUMO-CpdR was expressed directly from strain BPC223, a BL21(DE3) *E. coli* strain harboring plasmid pSUMO-His$_6$-CpdR (pET-23b derivative; *cpdR* 5’ fused to a his$_6$ tagged SUMO domain), as described above for the ChpT protein. His$_6$-SUMO-CpdR was expressed and purified from 5 L of BPC223 culture similar to reported methods (Abel et al., 2011). A soluble lysate was prepared by resuspending the harvested, frozen cell pellet in 50 mL lysis buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM DTT, 250U Benzonase nuclease (Sigma), SigmaFast protease inhibitor cocktail (Sigma)), lysing the cells with three passes through an Emulsiflex C5, 4°C, and removing the insoluble cell debris by centrifugation (29500 × *g*, 4°C, 30 min). His$_6$-SUMO-CpdR protein was affinity purified in batch: 8 mL 50% slurry of Ni-NTA agarose

affinity resin (5Prime) equilibrated in lysis buffer was incubated with the soluble, cleared lysate at 4°C for 90 min, the slurry was applied to a chromatography column, the resin bed was washed with 125 mL Ni-NTA wash buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM DTT) and the protein was eluted with 25 mL Ni-NTA elution buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 500 mM KCl, 250 mM imidazole, 10% (v/v) glycerol, 1 mM DTT). The His_6-SUMO tag was removed: to the eluted His_6-SUMO-CpdR was added 1 mL of 1.7 mg/mL SUMO-His_6 protease and the solution was dialyzed overnight at 4°C in two 12 mL Slide-A-Lyzer 10 k MWCO dialysis cassettes against 2L dialysis buffer (20 mM HEPES·KOH pH 8.0, 100 mM KCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT). The following day, the His_6-tagged proteins (uncut His_6-SUMO-CpdR, His_6-SUMO domain, and SUMO-His_6 protease) were removed by subtractive Ni-NTA affinity purification with 12 mL of 50% slurry of Ni-NTA agarose affinity resin (5Prime) equilibrated in dialysis buffer; the eluate containing cleaved CpdR was collected. The sample was concentrated to 6 mL by centrifuge ultrafiltration (3700 × *g*, 4°C) with a Vivaspin 20 concentrator 10k MWCO (GE Healthcare) and purified by size exclusion chromatography on a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) equilibrated in kinase storage buffer (50 mM HEPES·KOH pH 8.0 at 20°C, 200 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT) using a GP-250 FPLC gradient programmer (Pharmacia, now GE Healthcare) equipped with dual P-500 pumps (Pharmacia, now GE Healthcare) to yield approximately 12.6 mg of purified CpdR from 5 L of bacterial culture. We note that most of the expressed protein forms high molecular weight aggregates that we were able to separate by size exclusion chromatography. The eluted fractions at the expected molecular weight for monomeric CpdR were run on an analytical size exclusion column as described below to ensure CpdR was monomeric. CpdR protein was concentrated by centrifuge ultrafiltration (3700 × *g*, 4°C) using a Vivaspin 20 concentrator 10k MWCO (GE Healthcsare) to 9.7 mg/mL, flash frozen in liquid nitrogen and stored at –80°C. Protein purity was assessed to be >95% pure by Coomassie-stained polyacrylamide gel electrophoreses. The protein concentration was determined by measuring absorbance at 280 nm and using the calculated molar extinction coefficient of 5625 cm^{-1} M^{-1}. The calculated molecular weight of this construct is 12606 Da.

CtrA Cloning, Expression, and Purification—An expression plasmid for His_6-SUMO-CtrA was derived from the pSUMO-His_6-*cpdR* plasmid. *ctrA* gene was subcloned from pET28b-*ctrA* (Chien et al., 2007) using primers JABp144 and JABp145 to produce a *ctrA* with ligation independent cloning (LIC) overhangs reported by Weeks and co-workers (Weeks et al., 2007). Meanwhile, pSUMO-His_6-*cpdR* was reverse amplified with primers JABp142 and JABp143, which produced a linear fragment of the pSUMO-His_6 vector without *cpdR* and with complementary LIC overhangs (Weeks et al., 2007), and this PCR mixture was subsequently DpnI-treated to digest remaining pSUMO-His_6-*cpdR* plasmid DNA. Both the *ctrA* fragment and the linear pSUMO-His_6 vector fragment were purified by agarose gel electrophoresis and a QiaQuick gel extraction kit (Qiagen). These fragments were assembled by iso-thermal assembly using the Gibson method according to the protocols provided by the j5 manual (Hillson et al., 2012) (http://j5.jbei.org/j5manual/pages/79.html) and by Gibson *et al* (Gibson et al., 2009) to produce plasmid pJAB67, which was sequence-verified. Chemically competent Rosetta(DE3) pLysS *E. coli* were transformed with pJAB67 to produce expression strain JAB148. His_6-SUMO-CtrA was expressed and purified from JAB148 using methods described above for the purification of the His_6-ChpT variants.

Phosphotransfer assays—Phosphotransfer assays were performed as previously described (Biondi et al., 2006; Skerker et al., 2005). Briefly, phosphorylated CckA was generated by incubating 5 μM of His_6-$CckA_{70\text{-}691}$ in kinase buffer (50 mM HEPES·KOH pH 8.0, 200 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol, and 1 mM DTT) supplemented with 0.5 mM ATP, 0.167 μCi/μL [γ-^{32}P] ATP, 2 mM DTT, and 5 mM $MgCl_2$ at room temperature for 60 mins in a total reaction volume of 30 μL. Phosphotransfer from CckA to ChpT was measured by incubating 30 μL of 5 μM ^{32}P-phosphorylated His_6-$CckA_{70\text{-}691}$ with 30 μL of 5 μM His_6-ChpT or His_6-ChpT variants in kinase buffer supplemented with 5 mM $MgCl_2$. The reaction time was started upon addition of His_6-ChpT or the His_6-ChpT variants to the reaction mixture and allowed to proceed for 10s, at which time 8μL of the reaction mixture was removed and quenched into 2 μL of 5× Laemmli Sample Buffer. Each reaction was loaded onto 12% Tris-HCl gels for PAGE, the proteins separated by electrophoresis, the radioactivity in the wet gels recorded on a phosphor storage plate for 3 hrs, and the images were recorded on a Typhoon fluorescence imager (Molecular Dynamics). Images files were opened in ImageJ software (Schneider et al., 2012), converted to 8-bit images, cropped, and saved as 8-bit png files. Quantitation of band intensity was measured using ImageJ: rectangular regions were drawn around each ^{32}P-ChpT variant band and a region on the gel corresponding to

background intensity; the integrated intensities of these regions were measured and normalized to the wild-type ChpT band. Band intensities from three individual experiments were averaged, and the mean intensity and standard deviation were plotted using Prism 5 software (GraphPad).

For phosphorelay reactions, 2.5 μM His_6-$CckA_{70-691}$ was mixed with 2.5 μM His_6-SUMO-CtrA or 2.5 μM CpdR in kinase buffer (50 mM HEPES·KOH pH 8.0, 200 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol, and 1 mM DTT) supplemented with 0.5 mM ATP, 0.167 μCi/μL [γ-^{32}P] ATP, 1 mM DTT, and 5 mM $MgCl_2$ and allowed to incubate for 30 minutes. Then 8μL of the reaction mixture was removed and quenched into 2 μL of 5× Laemmli Sample Buffer Reaction. The quenched reaction products were separated via gel electrophoresis on 4–20% Tris-HCl gradient gels and analyzed as described above.

Analytical size exclusion chromatography—A gel filtration standard (Bio-rad) containing Thyroglobulin (bovine), γ-globulin (bovine), ovalbumin (chicken), myoglobulin (horse), and vitamin B12 were used to generate a molecular weight standard plot using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in kinase buffer (50 mM HEPES·KOH pH 8.0, 200 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol, and 1 mM DTT) and run with a 0.5 mL/min flow rate. Samples of His_6-ChpT and each variant (approximately 50 μL of a 2 mg/mL solution) were individually loaded onto the column and eluted. Each protein eluted at a volume corresponding to a His_6-ChpT dimer. Error bars for molecular weight estimated as the full width at half maximum (FWHM) for the eluting peak. The data are summarized in Table S1.

Construction of chpT::aacC1, chpT-mcherry *substitution variants*—To isolate strains whose sole copy of *chpT* harbored point mutations, we first constructed the mereodiploid strain JAB114 (*vanA::chpT-mcherry*) by transforming wild-type *C. crescentus* NA1000 (Marks et al., 2010) with pJAB51, which was made by amplifying *chpT* from plasmid pJAB30 using primers JABp72 and JABp113 and subcloning the resulting fragment at the NdeI and SacI sites into pVCHYC-2 (Thanbichler et al., 2007), an integrating vector for placing C-terminal proteins fused to *mcherry* at the *vanA* locus, generating a kanamycin-resistant strain. Next, we constructed pJK390, an allelic replacement vector to replace native *chpT* with the *aacC1* gentamycin-resistance cassette. We amplified 500 base pair fragments flanking the *chpT* open reading frame from the *C. crescentus* NA1000 genome—one fragment contained the 500 base pairs preceding the *chpT* start codon and was amplified using JWKP406 and JWKP407 and the other fragment contained the 500 base pairs succeeding the *chpT* stop codon and was amplified using JWKP410 and JWKP411—and the *aacC1*-containing gentamycin-resistance cassette from the pBXMCS-4 shuttle vector using JWKP408 and JWKP409. The primers used to generate these fragments were designed using the j5 software suite (Hillson et al., 2012) (http://j5.jbei.org/) to contain homology to the pNPTS138 vector (M. R. Alley, unpublished) as well as to one another to facilitate iso-thermal assembly using the Gibson method. Purified, BamHI-restricted pNPTS138 and the PCR-amplified fragments were incubated according to the protocols provided by the j5 manual (http://j5.jbei.org/j5manual/pages/79.html) and by Gibson *et al* (Gibson et al., 2009), and the reaction products—plasmid pJK390—were transformed into DH5α *E. coli*, generating strain JWK1428. Isolated colonies were then screened by PCR and proper assembly was confirmed by sequencing. To delete the native *chpT* allele in the genome of our *chpT* mereodiploid, we transformed JAB114 (*C. crescentus* NA1000 *vanA::chpT-mcherry*) with pJK390 and selected for gentamycin-resistant colonies. These isolates were then grown without selection and cells still containing integrated pJK390 (which contains the *sacB* open reading frame) were counter selected on media containing 3% sucrose and 0.5 mM vanillate. Individual colonies were then screened for the presence of the native *chpT* allele using the primers JWKP412 and JWKP413, yielding strain JWK1436 (*chpT::aacC1, vanA::chpT-mcherry*). This strain was then used to generate a ϕCr30 lysate, ϕ*chpT::aacC1*. Quikchange mutagenesis of pJAB51 was used to generate *chpT* variants containing point mutations in the DHp domain using methods described above for Quikchange mutagenesis of pJAB30. We isolated mereodiploid strains containing native *chpT* and *vanA::chpT-mcherry* S40V (JAB164), A41R (JAB165), S44Y (JAB166), G45R (JAB167), D47A (JWK1442), L48M (JWK1443), D60A (JWK1444), N63R (JWK1445), or L64D (JAB168) as before and then performed generalized transduction to generate *chpT::aacC1, vanA::chpT-mcherry* S40V (JWK1437), A41R (JWK1438), S44Y (JWK1439), G45R (JWK1440), D47A (JWK1450), L48M (JWK1451), D60A (JWK1452), N63R (JWK1453), or L64D (JWK1441).

Microscopy—chpT::aacC1, vanA::chpT-mcherry WT, S40V, A41R, S44Y, G45R, D47A, L48M, D60A, N63R, and L64D strains (JWK1436-JWK1441, JWK1450-1453) were cultured overnight in PYE supplemented

with 5 μM vanillate and sub-cultured into fresh media for six hours or until they reached an OD600 of 0.4. Cell suspensions were then dried onto agarose pads (1.5% agarose in PYE) containing 5 μM vanillate and imaged on a Leica DM 6000 B microscope with a HCX PL APO 100×/1.40 Oil PH3 CS objective, Hamamatsu EM-CCD C9100 camera and Metamorph (Molecular Devices). Both phase-contrast and fluorescence images were recorded. The fluorescent channel for mCherry was excited with a Leica EL6000 metal halide light source coupled via a liquid light guide, and the following excitation and emission filters were used: Ex 562/40 nm, Em 624/40 nm. ChpT overexpression microscopy studies of *chpT-mCherry* and variants were conducted as before, but with the addition of 0.5 mM vanillate to the PYE growth media.

Surface plasmon resonance (SPR) of CckA-ChpT interactions—SPR experiments were designed to measure CckA-RD binding to immobilized His_6-ChpT similar to methods described by Bell *et al*. (Bell et al., 2010). Purified His_6-ChpT, His_6-ChpT-A41R, and His_6-ChpT-G45R were immobilized on the Biacore CM5 sensor chips via an anti-His_6 antibody. The analyte used was the CckA receiver domain (CckA-RD, amino acids 571-691.) SPR experiments were performed on a Biacore 3000 system at 25°C using a flow rate of 30 μl min^{-1} in running buffer (50 mM HEPES·KOH pH 8.0, 200 mM KCl, 1 mM DTT). For each data set the signal in a control flow cell that lacked immobilized ligand was subtracted to account for non-specific binding and analyzed using the BIAevaluation software (Biacore).

Sequence Logos and Molecular modeling—We aligned ChpT, CckA, and CtrA alphaproteobacteria homologs (Brilli et al., 2010), extracted the core HK-RR co-varying interaction residues as determined for canonical HK-RR pairs (Capra et al., 2012a; Capra et al., 2012b; Skerker et al., 2008), and created sequence logos using Weblogo (Crooks et al., 2004; Schneider and Stephens, 1990). RD homology models of CckA, CtrA, and CpdR were built based on the RD template of an OmpR/PhoB homolog from *Thermotoga maritima* (Buckler et al., 2002) (PDB ID 1kgs). The template shares 38% sequence identities with the CtrA-RD using the WHAT IF program (Vriend, 1990), and the proposed ChpT recognition region (the α1 helix and the β4-α5 loop) are highly conserved in above models. For comparison, homology models were also generated using the I-TASSER server (Zhang, 2008). An initial molecular complex between ChpT and an RD was built based on the HK853/RR468 complex (Casino et al., 2009) (PDB ID 3dge) by superposing the RD to RR468 and ChpT onto HK853-DHp. The crude complexes were then optimized using the ROSETTA software (Das and Baker, 2008) by rigid-body and side-chains adjustments.

Circular dichroism of ChpT variants—The CD spectra of 8 μM His_6-ChpT substrates in 20 mM Tris·HCl pH 8.0, 50 mM NaCl were recorded at 25°C using a an AVIV Model 62A DS circular dichroism spectrometer (Aviv Associates, Lakewood, NJ) equipped with a Peltier temperature control unit using a 1 mm path length cuvette. CD spectra were the average of four scans from 190 to 260 nm with a step size of 2 nm, 1.5 nm band width, and signal averaged for 10 s. The protein concentration for each variant was determined by absorbance at λ=280 nm as described above for ChpT purification. Data were processed as described (Kelly et al., 2005).

SUPPLEMENTAL EXPERIMENTAL PROCEDURE TABLES

TABLE S1. ChpT and the ChpT variants are dimers in solution.

His_6-ChpT variant	Estimated MW gel filtration (kDa)	Calculated monomer MW (kDa)	Calculated dimer MW (kDa)
WT	60.7 ± 14.0	25.5	51.1
S40V	60.7 ± 6.6	25.5	51.1
A41R	64.1 ± 18.9	25.6	51.2
S44Y	57.5 ± 21.5	25.6	51.2
G45R	57.5 ± 21.5	25.6	51.3
D47A	54.4 ± 17.9	25.5	51.0
L48M	60.7 ± 15.6	25.5	51.1
D60A	60.7 ± 14.0	25.5	51.0
N63R	57.5 ± 25.3	25.6	51.1
L64D	60.7 ± 25.3	25.5	51.1

TABLE S2. Plasmids used in this study.

Plasmid	Description	Reference
pET-28b(+)	Bacterial expression vector encoding an N-terminal His_6-tag and a thrombin cleavage site, Kan^R	Novagen
pJAB27	pET28b-*cckA*$_{70\text{-}691}$, Kan^R	This study
pJAB28	pET28b-*cckA*$_{571\text{-}691}$, Kan^R	This study
pJAB30	pET28b-*chpT*, Kan^R	This study
pJAB35	pET28b-*cckA*$_{71\text{-}691}$-H322A, Kan^R	This study
pJAB36	pET28b-*cckA*$_{70\text{-}691}$-H322A, Kan^R	This study
pJAB51	pVCHYC2-*chpT*, Kan^R	This study
pJAB53	pET28a-*chpT*-S40V, Kan^R	This study
pJAB54	pET28a-*chpT*-A41R, Kan^R	This study
pJAB55	pET28b-*chpT*-S44Y, Kan^R	This study
pJAB56	pET28b-*chpT*-G45R, Kan^R	This study
pJAB57	pET28a-*chpT*-D47A, Kan^R	This study
pJAB58	pET28b-*chpT*-L48M, Kan^R	This study
pJAB59	pET28b-*chpT*-D60A, Kan^R	This study
pJAB60	pET28b-*chpT*-N63R, Kan^R	This study
pJAB61	pET28b-*chpT*-L64D, Kan^R	This study
pJAB67	pSUMO-LIC-*ctrA*, Amp^R	This study
pJAB77	pVCHYC2-*chpT*-S40V, Kan^R	This study
pJAB78	pVCHYC2-*chpT*-A41R, Kan^R	This study
pJAB79	pVCHYC2-*chpT*-S44Y, Kan^R	This study
pJAB80	pVCHYC2-*chpT*-G45R, Kan^R	This study
pJAB81	pVCHYC2-*chpT*-L64D, Kan^R	This study
pJAB82	pVCHYC2-*chpT*-D47A, Kan^R	This study
pJAB83	pVCHYC2-*chpT*-L48M, Kan^R	This study
pJAB84	pVCHYC2-*chpT*-D60A, Kan^R	This study

Plasmid	Description	Reference
pJAB85	pVCHYC2-*chpT*-N63R, Kan^R	This study
pNJH87	pMT383-*cckA* (contains *cckA* (CC_1078) sequence cloned from CB15N genomic DNA at NdeI & EcoRI sites), Kan^R	(N. Hillson, unpublished)
pWSC19	pET28b-FLAG-$cckA_{70\text{-}691}$, Kan^R	This study
pNTPS138	Suicide vector containing *sacB* for gene deletions, Kan^R	(M. R. Alley, unpublished)
pVCHYC-2	pP_{van}-*mcherry* expression integration vector, Kan^R	(Thanbichler and Shapiro, 2006)
pSUMO-His_6-*cpdR*	pET-23b derivative; *cpdR* 5' fused to a his_6 tagged SUMO domain, Amp^R, gift from Peter Chien (UMass Amherst)	(Abel et al., 2011)
pET23b-*ulp1*	SUMO protease catalytic domain from *ulp1* cloned into pET-23b, Amp^R, gift from Peter Chien (UMass Amherst)	(Wang et al., 2007)

TABLE S3. Strains used in this study.

Strain	Description	Reference
C. crescentus NA1000	Laboratory *Caulobacter crescentus* strain	(Marks et al., 2010)
E. coli DH5α	Bacterial cloning strain	Invitrogen
E. coli Rosetta(DE3)pLysS	Bacterial expression strain	Novagen
JAB70	*E. coli* Rosetta(DE3)pLysS + pJAB27[pET28b-$cckA_{70\text{-}691}$]	This study
JAB73	*E. coli* Rosetta(DE3)pLysS + pJAB28[pET28b- $cckA_{571\text{-}691}$]	This study
JAB75	*E. coli* Rosetta(DE3)pLysS + pJAB30[pET28b-*chpT*]	This study
JAB90	*E. coli* Rosetta(DE3)pLysS + pJAB36[pET28b-$cckA_{70\text{-}691}$-H322A]	This study
JAB134	*E. coli* Rosetta(DE3)pLysS + pJAB53[pET28a-*chpT*-S40V]	This study
JAB135	*E. coli* Rosetta(DE3)pLysS + pJAB54[pET28a-*chpT*-A41R]	This study
JAB136	*E. coli* Rosetta(DE3)pLysS + pJAB55[pET28b-*chpT*-S44Y]	This study
JAB137	*E. coli* Rosetta(DE3)pLysS + pJAB56[pET28b-*chpT*-G45R]	This study
JAB138	*E. coli* Rosetta(DE3)pLysS + pJAB57[pET28a-*chpT*-D47A]	This study
JAB139	*E. coli* Rosetta(DE3)pLysS + pJAB58[pET28b-*chpT*-L48M]	This study
JAB141	*E. coli* Rosetta(DE3)pLysS + pET23b-*ulp1*	This study; (Wang et al., 2007)
JAB143	*E. coli* Rosetta(DE3)pLysS + pJAB59[pET28b-*chpT*-D60A]	This study
JAB144	*E. coli* Rosetta(DE3)pLysS + pJAB60[pET28b-*chpT*-N63R]	This study
JAB145	*E. coli* Rosetta(DE3)pLysS + pJAB61[pET28b-*chpT*-L64D]	This study
JAB148	*E. coli* Rosetta(DE3)pLysS + pJAB67[pSUMO-LIC-*ctrA*]	This study
JWK1428	*E. coli* DH5α + pJK390[pNTPS138(*chpT* UP, gentR, DN)]	This study
JWK1430	*C. crescentus* NA1000 *vanA::chpT-mcherry* WT	This study
JWK1431	*C. crescentus* NA1000 *vanA::chpT-mcherry* S40V	This study
JWK1432	*C. crescentus* NA1000 *vanA::chpT-mcherry* A41R	This study
JWK1433	*C. crescentus* NA1000 *vanA::chpT-mcherry* S44Y	This study
JWK1434	*C. crescentus* NA1000 *vanA::chpT-mcherry* G45R	This study
JWK1435	*C. crescentus* NA1000 *vanA::chpT-mcherry* L64D	This study
JWK1436	*C. crescentus* NA1000 *vanA::chpT-mcherry* WT, *chpT::aacC1*	This study
JWK1437	*C. crescentus* NA1000 *vanA::chpT-mcherry* S40V, *chpT::aacC1*	This study
JWK1438	*C. crescentus* NA1000 *vanA::chpT-mcherry* A41R, *chpT::aacC1*	This study

Strain	Description	Reference
JWK1439	*C. crescentus* NA1000 *vanA::chpT-mcherry* S44Y, *chpT::aacC1*	This study
JWK1440	*C. crescentus* NA1000 *vanA::chpT-mcherry* G45R, *chpT::aacC1*	This study
JWK1441	*C. crescentus* NA1000 *vanA::chpT-mcherry* L64D, *chpT::aacC1*	This study
JWK1446	*C. crescentus* NA1000 *vanA::chpT-mcherry* D47A	This study
JWK1447	*C. crescentus* NA1000 *vanA::chpT-mcherry* L48M	This study
JWK1448	*C. crescentus* NA1000 *vanA::chpT-mcherry* D60A	This study
JWK1449	*C. crescentus* NA1000 *vanA::chpT-mcherry* N63R	This study
JWK1450	*C. crescentus* NA1000 *vanA::chpT-mcherry* D47A, *chpT::aacC1*	This study
JWK1451	*C. crescentus* NA1000 *vanA::chpT-mcherry* L48M, *chpT::aacC1*	This study
JWK1452	*C. crescentus* NA1000 *vanA::chpT-mcherry* D60A, *chpT::aacC1*	This study
JWK1453	*C. crescentus* NA1000 *vanA::chpT-mcherry* N63R, *chpT::aacC1*	This study
BPC223	*E. coli* BL21(DE3) pLysS + pSUMO-His_6-*cpdR*, gift from Peter Chien (UMass Amherst)	(Abel et al., 2011)
BPC250	*E. coli* DH5α + pET23b-*ulp1*, gift from Peter Chien (UMass Amherst)	(Wang et al., 2007)

TABLE S4. Oligonucleotides used in this study.

Primer	Description	Primer Sequence (5'–3')
JABp64	cckA-70_f	GGCCTTGTCcatATgCGCGGCTCAGCGCTTTCCGG
JABp65	cckA-571_f	AGGAgctctCaGGCGCCCGACAGGTCGCGAGCG
JABp66	cckA-691_r	ACTGCgGAgcTCCTACGCCGCCTGCAGCTGCTG
JABp68	amp_chpT_f	CCAAGCataTGCGTTATTTTCTCCAAGCGC
JABp69	amp_chpT_r	TgAgctcTTACGCCGGGACCCAG
JABp72	chpT_M29_f	CGCCcatATGACCGAGACCGTC
JABp85	pET28-SacI-megapri_f	CAGCCGGATCTCAGTG
JABp86	pET28-NcoI-megapri_r	TCGAGATCTCGATCCCGCGAAATTAATACGACTCACT
JABp88	cckA-H322A-megari2_r	TCGCGgcCGACTTCAA
JABp113	amp_chpT-M29	ACGGAGagCTcgCGCCGGGACC
JABp115	chpT_S40V_1	CCCGAGACGATGGCGACGGCCGGACTGATGAAG
JABp116	chpT_S40V_2	CTTCATCAGTCCGGCCGTCGCCATCGTCTCGGG
JABp117	chpT_A41R_1	CAGGCCCGAGACGATGCGGCTGGCCGGACTGATG
JABp118	chpT_A41R_2	CATCAGTCCGGCCAGCCGCATCGTCTCGGGCCTG
JABp119	chpT_S44Y_1	CAGCAGATCCAGGCCATAGACGATGGCGCTGG
JABp120	chpT_S44Y_2	CCAGCGCCATCGTCTATGGCCTGGATCTGCTG
JABp121	chpT_G45R_1	CCAGCAGATCCAGGCGCGAGACGATGGCGC
JABp122	chpT_G45R_2	GCGCCATCGTCTCGCGCCTGGATCTGCTGG
JABp123	chpT_D47A_1	GGTCTTCCAGCAGAGCCAGGCCCGAGACG
JABp124	chpT_D47A_2	CGTCTCGGGCCTGGCTCTGCTGGAAGACC
JABp125	chpT_L48M_1	GGGGTCTTCCAGCATATCCAGGCCCGAGAC
JABp126	chpT_L48M_2	GTCTCGGGCCTGGATATGCTGGAAGACCCC
JABp127	chpT_D60A_1	GATCAGGTTCATGGCGGCGTCGCGCATGTCCTG
JABp128	chpT_D60A_2	CAGGACATGCGCGACGCCGCCATGAACCTGATC
JABp129	chpT_N63R_1	CGAGGAGGCGATCAGACGCATGGCGTCGTCGC
JABp130	chpT_N63R_2	GCGACGACGCCATGCGTCTGATCGCCTCCTCG
JABp131	chpT_L64D_1	GGCCGAGGAGGCGATATCGTTCATGGCGTCGTCG

Primer	Description	Primer Sequence (5'–3')
JABp132	chpT_L64D_2	CGACGACGCCATGAACGATATCGCCTCCTCGGCC
JABp142	revamp_pSUMOcpdR_1	CCACCaaTCTGTTCTCTGTGAGCCTC
JABp143	revamp_pSUMOcpdR_2	tctaaActctcctCgagAAGCTTGCGGCC
JABp144	LIC-Loll_ctrA_f	agattggtggcCGCGTACTGTTGATCGAGGATGAC
JABp145	LIC-Loll_ctrA_r	gaggagagtttagacTCAGGCGGCGTTAACCTGCTC
JWKP406	Gibson_500bp-chpT_1	AAGCTTCTCTGCAGGATATCTGGATCAGGCGCTCGCCGGC GCGAT
JWKP407	Gibson_500bp-chpT_2	GTAAATTGTCACAAGGCGGCGAACTCTGCCGTGATTT
JWKP408	Gibson_pBXMCS-4_1	CAGAGTTCGCCGCCTTGTGACAATTTACCGAACAACTC
JWKP409	Gibson_pBXMCS-4_2	CGGAGATTGTTAAAGATTGCGTATAATATTTGCCCATGGA C
JWKP410	Gibson_500bp-chpT_3	GGGCAAATATTATACGCAATCTTTAACAATCTCCGCGCC
JWKP411	Gibson_500bp-chpT_4	GTCACGGCCGAAGCTAGCGAATTCGTGCGGGCGCGGCCC AGGGCC
JWKP412	chpT_seq_1	ACCTGATCGAGAGCCGGC
JWKP413	chpT_seq_2	ATCCCGGTGATCTCGG
WSCp29	FLAG-cckA_1	gaataaCataTggactataagGacGaCGacGacaaGTcaGG-CggcGgCGgCtcaCGCGGCTCAGCGCTTTCC
WSCp30	FLAG-cckA_2	ACCGTCGACTGCAGAgcTCCTACGCCGCCTGCAGCTGCTG CTTG-ACGC

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