Appendix / Supplementary Information

Transmembrane redox control and proteolysis of PdeC, a novel type of c-di-GMP phosphodiesterase

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Supplementary Materials & Methods

Bacterial strains

All strains used are derivatives of the *E. coli* K-12 strain W3110 (Hayashi et al., 2006). The *pdeB(ylaB)::cat*, *pdeC(yjcC)::kan*, *pdeD(yoaD)::cat*, *pdeG(ycgG)::kan, pdeH(yhjH)::kan*, *pdeN(rtn)::kan* and *csgB::kan* mutations are full ORF deletion(Δ)/resistance cassette insertions previously described (Sommerfeldt et al., 2009). For generating the *cya::kan, degP::kan*, *degQ::kan, dsbA::kan, dsbB::kan, dsbC::kan, dsbD::kan, dsbG::kan and* Δ(*lacI-A*)::*kan* full ORF deletion/insertion mutants, the one-step-inactivation protocol was applied (Datsenko and Wanner, 2000) using oligonucleotide primers listed in Table S1. Mutations associated with resistance cassettes were transferred by P1 transduction (Miller, 1972). When required (e.g. for combining multiple mutations), cassettes were flipped out (Datsenko and Wanner, 2000), which is designated as '*::scar'*. *ΔrpoS*, *ΔbcsA* and *ΔcsgD* are such scarred derivatives of previously described full ORF deletion/*cat* cassette insertions (Pesavento et al., 2008; Serra et al., 2013; Weber et al., 2006). Strains carrying the previously described single copy *csgB*::*lacZ* reporter fusion (inserted at the att(λ) site) (Weber et al., 2006) also carried a Δ(*lacI-A*)::*scar* deletion.

The introduction of point mutations into chromosomal genes was performed with a two-step method related to the one-step gene inactivation (Kolmsee and Hengge, 2011). All oligonucleotide primers are listed below in Table S1.

Growth of bacterial macrocolonies

Cells were grown overnight in liquid LB medium (Miller, 1972) under aeration at 37°C. 5 µl of the overnight cultures were spotted on salt-free LB or YESCA agar plates (the latter containing 10 g/l casamino acids instead of tryptone as in LB). Agar plates were supplemented with Congo Red (CR, 40 µg ml^{-1}) and Coomassie brilliant blue (20 µg ml^{-1}) ('CR plates'). For growing plasmid-containing strains, 100 µg ml^{-1} ampicillin was included in the agar plates. In order to grow all strains to be compared in parallel on a single agar plate, 140 mm-diameter Petri dishes (VWR) were used, which allows the spotting of up to 25 macrocolonies in a 5x5 array on a single plate. Plates with macrocolonies were incubated at 28°C for 5 days.

Construction of low copy number plasmids expressing wildtype and mutant variants of PdeC

The plasmids used in this study were constructed using the low copy number vector pCAB18, which carries the *tac* promoter followed by a multiple cloning site (Barembruch and Hengge, 2007). C-terminal His6 tags fused to the PdeC variants were introduced via the oligonucleotide primers used for PCR. Oligonucleotides used in this study are listed in Table S1 (see below). Point mutations were introduced by a two-step-PCR protocol. Four primers were used, with two introducing the mutation and the others flanking the up- and downstream regions of the gene and carrying restriction sites for cloning into pCAB18. In the first step, two separate PCR fragments were generated that overlap in the region of the mutation and cover the up- and downstream regions, respectively. In the second step these fragments were used in combination as templates for a PCR reaction with the up- and downstream primers only in order to generate the final PCR fragment. This fragment contains the entire gene carrying the mutation flanked by the restriction sites for cloning, which was then inserted into the vector. A similar two-step PCR protocol was used to generate the pdeC/N chimeric construct (consisting of TM1-CSS domain-TM2 of PdeC followed by the EAL domain of PdeN) with the exception that the two 'internal' primers used in the first PCR reactions determined the fusion site.

Cloning of pdeC$^{\Delta peri}$, i.e. the PdeC variant that lacks the region encoding the periplasmic domain of PdeC (from T44 to R234), was performed by using a fully synthetic DNA fragment (obtained from GeneArt® Strings, Invitrogen) that also contained the restriction sites necessary for digestion and ligation into the vector pCAB18.

For replacing transmembrane regions TM1 and/or TM2 in PdeC (expressed from pCAB18) by the first and/or second transmembrane segments of LacY (see Fig. S2 for exact amino acid sequences substituting for each other), synthetic DNA fragments (obtained from GeneArt® Strings, Invitrogen) were used (for sequences, see Table S2). The fragments contained either the entire *pdeC* gene or only the 5´-region of *pdeC* (both with the necessary sequence substitutions). The fragments were amplified by PCR, thereby also introducing the EcoRI site directly upstream of the *pdeC* start codon (in a similar configuration also used for cloning of all other pdeC alleles). The subsequent cloning into pCAB18 made use of this *Eco*RI site as well as of the *Eag*I and *Eco*RV sites naturally located between and after the DNA regions encoding the two transmembrane regions, respectively. The replacement of TM2 by TM2* in various PdeC hybrid proteins expressed from pKNT25 and pUT18 essentially followed a two-step PCR mutagenesis protocol as described above (for primer sequences, see Table S1). For the first two PCR reactions, primer pairs *pdeC*^{wt}-XbaI-for/TM2*-rev and TM2*-for/*pdeC*^{wt}-SmaI-rev were used and the resulting two DNA fragments (overlapping in the TM2* region)

were used for the second step PCR with the 'external' primer pair *pdeC*^{wt}-XbaI-for/ *pdeC*^{wt}-SmaI-rev to generate the full size *pdeC*$^{wt/TM2*}$ or *pdeC*$^{ASS/TM2*}$fragments to be cloned into pKNT25 and pUT18. For cloning the region encoding TM2*+EAL into pKNT25 and pUT18, the primer pair *pdeC*^{TM2*EAL}-XbaI-for/ *pdeC*^{wt}-SmaI-rev was used, with the former encoding a new N-terminus followed by the complete TM2* and the six codons of pdeC that follow its natural TM2.

The PdeC$^{EAL\Delta C}$ variant (containing C285S, C299S and C489S exchanges), was generated by using a synthetic DNA fragment (obtained from GeneArt® Strings, Invitrogen) that contained the 3´-region of *pdeC* with mutations resulting in these cysteine replacement and followed by a His6 tag-encoding region. This fragment was used to replace the corresponding region in the pCAB18 derivative already carrying *pdeC*^{wt}, by making use of the natural *Eag*I restriction site in *pdeC* and the *Hin*dIII restriction site downstream of *pdeC* (originating from the multiple cloning site) were used.

Detection of protein-protein interaction in vivo using a bacterial two-hybrid system.

In order to detect *in-vivo* dimerisation of proteins and interactions with other proteins, the adenylate cyclase-based bacterial two-hybrid system was used (Karimova et al., 1998). This approach is based on the reconstitution of adenylate cyclase (CyaA) activity from two fragments (T25, T18) fused to two potentially interacting proteins. The hybrid proteins were expressed from plasmids (derived from the vectors pKNT25 and pUT18) that were transformed into *Δcya::scar* or *Δcya::scar ΔdegP::scar ΔdegQ::scar* derivatives of *E.coli* K-12 strain W3110. These *cya* mutants are unable to produce cAMP and utilize maltose as a carbon source on MacConkey agar base medium (white colonies). Interaction of the plasmid-encoded hybrid proteins in co-transformants generates a *cya*$^{+}$ phenotype (red colonies; these were obtained by spotting 5 µl of a 50 µl liquid suspension in 0.9 % NaCl of co-transformants onto MacConkey plates supplemented with 1% maltose, 100 µl/ml ampicillin and 50 µl/ml kanamycin and growth for 24-48 h growth at 28°C). Only when indicated, 0.05mM IPTG was added. In all constructs used in this study, the T25 and T18 fragments were fused to the C-termini of the PdeC protein variants.

SDS polyacrylamide gel electrophoresis and immunoblot detection

For immunoblot analyses samples were taken in the postexponential phase (pe; i.e. at an OD_{578} of approximately 3) and from overnight cultures grown in LB medium. Samples corresponding to 120 µg of total cellular protein were precipitated with 10% trichloracetic

acid (TCA). The protein pellets were resuspended in 60 µl of SDS-PAGE sample buffer without DTT and incubated for 10 min at 70°C and 95°C, respectively. Samples (15 µl) were run on 12% SDS-polyacrylamid gels. In cases where the apparent sizes of proteins had to be compared, total protein content of the samples was adjusted to yield similar amounts of the relevant proteins. Proteins were detected by immunoblotting as previously described (Lange and Hengge-Aronis, 1994) using antibodies against CsgD (custom-made by Pineda Antikörper Service), His6-tag (Bethyl Laboratories, Inc.) or Flag tag (Sigma). Anti-rabbit or anti-mouse IgG horseradish-peroxidase conjugate from donkey (GE Healthcare) was used for protein visualization in the presence of Western Lightning® Plus-ECL enhanced chemiluminescence substrate (PerkinElmer). Standard protein size markers were obtained from Thermo Scientific.

Separation of soluble and membrane-associated proteins

In order to obtain soluble and membrane fractions of cellular extracts, 200 µl of bacterial cultures grown overnight at 28°C were added to 1 ml 0.1 M NaOH (freshly diluted from a 10 M stock solution). Further sample preparation was performed according to an established protocol (Russel and Model, 1982).

Overproduction and purification of PdeC variants and other proteins.

C-terminally His6-tagged versions of $PdeC^{wt}$, $PdeC^{EAL\Delta C}$ and PdeC/N were purified from pCAB18-derived plasmids in the W3110 strain carrying *degP*::scar, *degQ*::scar double mutations in order to minimize the proteolytic procession of the desired purified proteins. Strains were grown in LB/ampicilline at 28°C to an OD_{578} of 0.5 to 0.7, IPTG (0.1 mM) was added and incubation continued for 5h at 28°C. Cells were harvested and lysed by three passages through a French press. Membrane proteins were purified essentially as described (Hebbeln et al., 2007) with minor variations. Cell debris was pelleted by centrifugation at 5000 rpm for 20 min. Membranes were pelleted by ultracentrifugation at 36000 rpm for 90 min, resuspended and homogenized in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 300 mM NaCl with protease inhibitor (cOmplete Tablets, Roche) and solubilized in the presence of 2 % (wt/vol) n-dodecyl β-D-maltoside (DDM; Sigma Aldrich) and 5 % (vol/vol) glycerol under agitation at 4°C for 2 hours. Nonsolubilized material was pelleted by ultracentrifugation. The supernatant containing the solubilized membrane fraction was used for protein purification. Imidazole was added to a final concentration of 20 mM and the solution was incubated with Ni-NTA (Protiono Ni-NTA Agarose, Macherey-Nagel) overnight. The whole solution was

transferred onto chromatography columns (Bio Rad) and washed with 50 mM Tris-HCl (pH 7.5) containing 0.05% DDM, 5% glycerol, 300 mM NaCl, 10 mM $MgCl_2$, protease inhibitor and imidazole up to 20 mM. Proteins were eluted by addition of 250 mM imidazole. For the PDE-assay the buffer was exchanged to 25 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 100 mM NaCl and 5 % glycerol using PD-10 columns (GE Healthcare). Purified protein was concentrated by Amicon Ultra-4 centrifugal filters (30000 NMWL; Merck Millipore). Relative approximate concentrations of the purified PdeC and its variants were controlled by SDS polyacrylamide gel electrophoresis with marker proteins at standardized concentrations (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis, GE Healthcare). Samples were stored at -80°C.

The soluble *E. coli* PDE PdeH was purified as previously described (Lindenberg et al., 2013) and was used for producing the control spot of radiolabeled pGpG for thin-layer chromatography.

Reconstitution of PdeC and PDE activity assays in lipid bilayer nanodiscs.

For the reconstitution of His6-tagged variants of PdeC into nanodiscs, we followed previously published protocols (Inagaki et al., 2013; Ritchie et al., 2009) with some variations. 12.5 mg of chloroform-solubilized *E. coli* total lipid extract (Avanti Polar Lipids) was vacuum dried. Lipids were subsequently hydrated by the addition of ND buffer (20 mM Tris pH 7.5; 100 mM NaCl) and DDM (0.01 % final concentration) followed by 5-10 min of sonification. Purified membrane scaffold protein MSP1E3D1 and purified PdeC, $PdeC^{EAL\Delta C}$ and PdeC/N, respectively, were added to the solubilized lipids, resulting in equimolar ratios of between scaffold protein and the PdeC variants (all at final concentrations of 3 μM). Samples were incubated for 1 h at 4°C and the detergent was removed using 1.5 g wet SM-2 Biobeads (Biorad, equilibrated in ND buffer) per 5 ml volume. As the membrane protein storage buffer contains 10% glycerol, which interferes with nanodisc-formation, ND buffer was added until glycerol concentration was below 3%. To prevent lipid-oxidation, samples were covered with N_2 gas. After 2 h of incubation at 4°C, ND buffer containing the nanodiscs was separated from the beads and incubated with Ni-NTA agarose (Protiono Ni-NTA Agarose, Macherey-Nagel) overnight. Purification of nanodiscs with incorporated PdeC variants was performed as described for purification of PdeC proteins except for the absence of detergent and protease-inhibitor. Purified nanodiscs were concentrated by Amicon Ultra-4 centrifugal filters (10000 NMWL; Merck Millipore). Relative approximate concentrations of the scaffold protein and PdeC in the nanodiscs were controlled by SDS polyacrylamide gel electrophoresis with

marker proteins at standardized concentrations (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis, GE Healthcare). Ratios between PdeC variants and scaffold protein (two molecules of which are required to form a nanodisc) varied between 0.5 and 1, i.e. preparations contained a mixture of nanodiscs with one and two molecules of the PdeC variants. Nanodisc preparations were stored at 4°C no longer than 7 days.

PDE activity of PdeC and its variants reconstituted in nanodiscs were performed as described (Weber et al., 2006) with [α-^{32}P]-c-di-GMP (Hartmann Analytic) as a substrate. Protein concentration in the assay was 57 nM and, where indicated, 6.5 mM DTT was added to the reactions. Samples were taken at 30, 60, 90 and 120 min and were analysed by thin layer chromatography and phosphoimaging (Thyphoon FLA-7000, Fuji). To generate the control spot of 5´-pGpG on thin-layer chromatographs, the PDE assay was performed with a purified soluble PDE, i.e. PdeH from *E. coli* K-12 (900 nM in the assay, 30 min reaction time).

Cryosectioning of macrocolony biofilms and fluorescence microscopy.

The procedure and materials used for cryomicrotomy of macrocolony biofilms and to examine and visualize the fluorescence of Thioflavin S (TS), which binds to both amyloid curli fibres and cellulose, in cryosections have been described in detail (Serra and Hengge, 2017). Briefly, cryomicrotomy was performed using an HM560 Cryostat (Thermo Fisher Scientific) set at -20 °C. Disposable Sec35 blades (Thermo Fisher Scientific) were used to obtain five-micrometer-thick sections perpendicular to the plane of macrocolonies. ProLong 9 Gold antifade reagent (Invitrogen, Carlsbad, CA) was added to the slides as a mounting medium. For fluorescence microscopy, an Axio Observer.Z1 microscope (Zeiss) was used with the following filter configurations for Thioflavin S: excitation = 436 bp/20nm and emission = 535 bp/30nm. Samples were visualized at 630X magnification. All digital images were captured using an AxioCam 506 mono digital camera coupled to the Axio Observer.Z1 microscope and Zen 2 software (Zeiss). When indicated, fluorescence images were superimposed with the corresponding phasecontrast images to show the location of fluorescence on the macrocolony sections. All cryosectioning and fluorescence microscopy experiments were done in two biological replicates.

Supplementary Figures

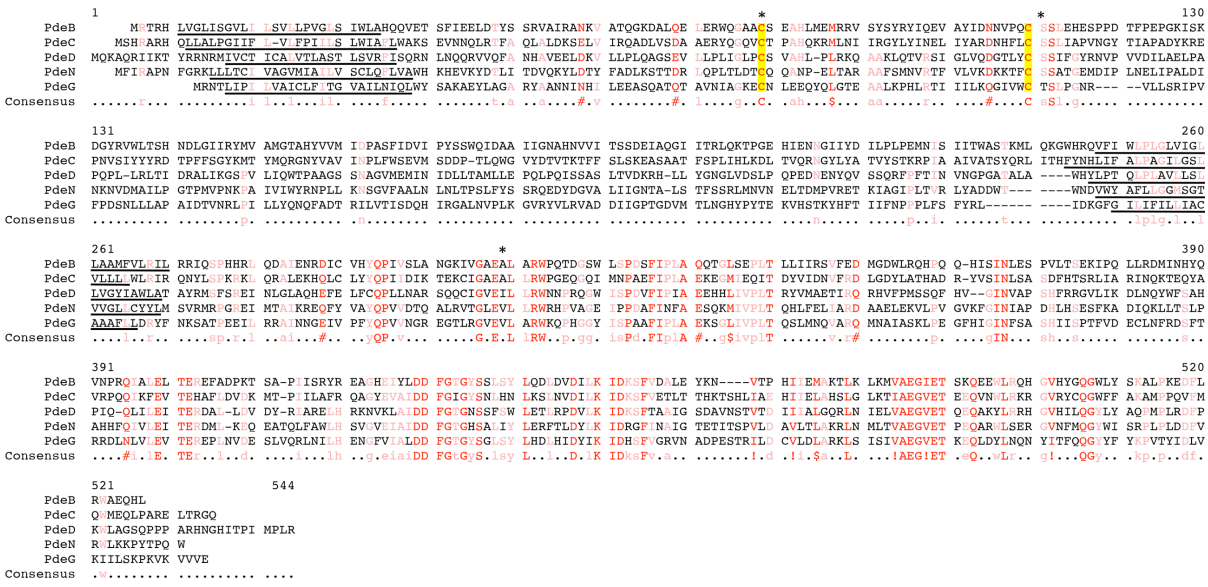

Fig. S1: Amino acid sequence alignment of the five CSS domain PDEs of *E. coli* K-12. Amino acid sequences of PdeB, PdeC, PdeD, PdeN and PdeG were aligned using the MultAlin algorithm (Corpet, 1988). Highly conserved amino acids are shown in red, less but still significantly conserved amino acids in light red. The two functionally relevant cysteine residues in the periplasmic loop, with the second one being part of the highly conserved CSS motif, are highlighted in yellow. Hydrophobic regions corresponding to the two transmembrane domains (TM1, TM2) flanking the periplasmic CSS domain are underlined. Asterisks denote positions where point mutations in PdeC were introduced in this study.

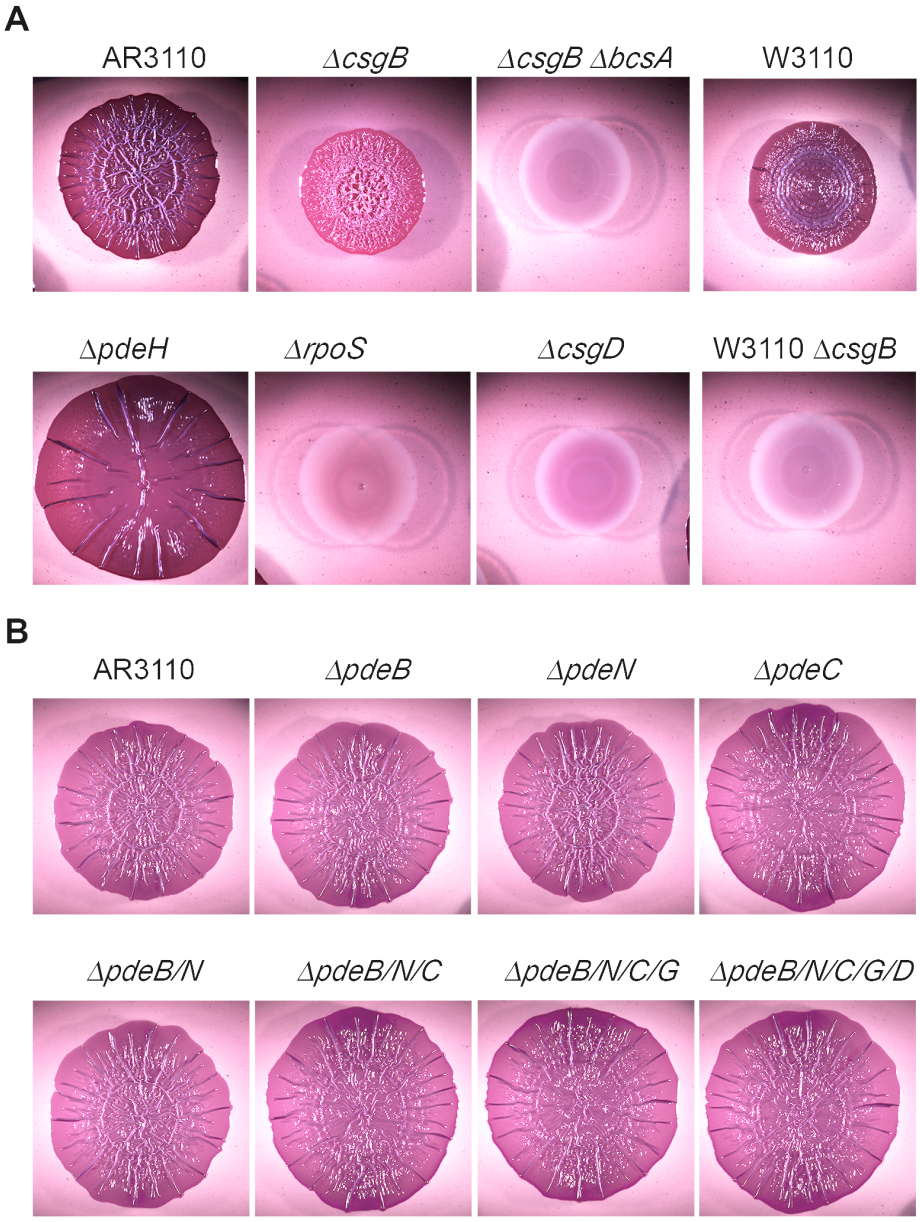

Figure S2. Macrocolony morphology as an indicator of biofilm matrix composition.
A: The *E. coli* K-12 strain AR3110 produces curli fibres as well as cellulose which results in large flat macrocolonies with ridges and wrinkles. Characteristic changes of macrocolony morphology are observed in strains that do not produce curli fibres (AR3110 *ΔcsgB*) or cellulose (W3110) or in the absence of the major cellular PDE PdeH (YhjH). The increased cellular c-di-GMP level in the *pdeH* mutant leads to increased expression of the biofilm regulator CsgD, which results in enhanced biosynthesis of both curli and cellulose, which generates very flat and stiff macrocolonies that only occasionally buckle up into long and very high ridges. Mutants that lack the RpoS sigma subunit of RNA polymerase or CsgD as well as a W3110 derivative in which also curli production is eliminated are completely devoid of extracellular matrix components and form white unstructured macrocolonies just as the *csgB bcsA* double mutant.
B: Macrocolony morphology of the AR3110 derivatives carrying single or multiple knockout mutations in genes encoding CSS domain-containing PDEs.

PdeC

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1     MSHRARHQLL ALPGIIFLVL FPIILSLWIA FLWAKSEVNN QLRTFAQLAL   50
51    DKSELVIRQA DLVSDAAERY QGQVCTPAHQ KRMLNIIRGY LYINELIYAR  100
101   DNHFLCSSLI APVNGYTIAP ADYKREPNVS IYYYRDTPFF SGYKMTYMQR  150
151   GNYVAVINPL FWSEVMSDDP TLQWGVYDTV TKTFFSLSKE ASAATFSPLI  200
201   HLKDLTVQRN GYLYATVYST KRPIAAIVAT SYQRLITHFY NHLIFALPAG  250
251   ILGSLVLLLL WLRIRQNYLS PKRKLQRALE KHQLCLYYQP IIDIKTEKCI  300
301   GAEALLRWPG EQGQIMNPAE FIPLAEKEGM IEQITDYVID NVFRDLGDYL  350
351   ATHADRYVSI NLSASDFHTS RLIARINQKT EQYAVRPQQI KFEVTEHAFL  400
401   DVDKMTPIIL AFRQAGYEVA IDDFGIGYSN LHNLKSLNVD ILKIDKSFVE  450
451   TLTTHKTSHL IAEHIIELAH SLGLKTIAEG VETEEQVNWL RKRGVRYCQG  500
501   WFFAKAMPPQ VFMQWMEQLP ARELTRGQ
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LacY (N-terminus only)

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1     MYYLKNTNFW MFGLFFFFYF FIMGAYFPFF PIWLHDINHI SKSDTGIIFA   50
51    AISLFSLLFQ PLFGLLSDKL GLRK...
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Figure S3. Complete amino acid sequence of PdeC and relevant sequence of LacY. The transmembrane segments of PdeC, i.e. TM1 (Q8 – A34) and TM2 (L235 – L262) as well as the N-terminal two transmembrane segments of LacY (N8 – L34) and (G46 – S67) are highlighted in grey. In various PdeC derivatives TM2 was precisely replaced by the second transmembrane segment of LacY (termed TM2*). The amino acids eliminated by a large deletion of the periplasmic loop domain in PdeC$^{\Delta peri}$ are underlined. Cysteine residues in PdeC are highlighted in yellow, with C75 and the CSS motif in the periplasmic loop also highighted in boldface. The EAL motif characteristic for PDEs is highlighted in red. Basic and acidic amino acids are labeled in blue and red, respectively.

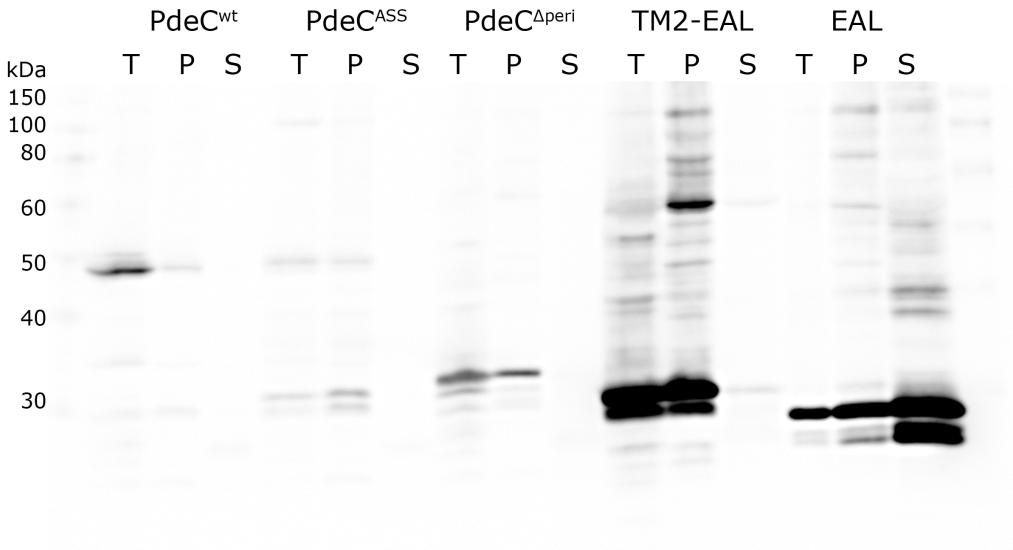

Figure S4. Association of the different PdeC variants and fragments with membrane and soluble cell fractions. Derivatives of strain W3110 expressing PdeC variants with the indicated mutations from pCAB18-derived plasmids were grown in LB medium at 28ºC. Samples corresponding to 80 μg total cellular protein were taken from overnight cultures. After NaOH treatment (see Supplemental methods described above), total samples (T) were fractionated into membrane protein-containing pellet (P) and soluble protein-containing supernatant (S) fractions, with proteins visualized by SDS-PAGE and immunoblotting (making use of the C-terminal His6 tag of all constructs). Note that the minor fraction of the soluble EAL domain that is found in the pellet fraction, most likely represents protein present in a minority of whole cells not destroyed by NaOH treatment that are found in the pellet fraction.

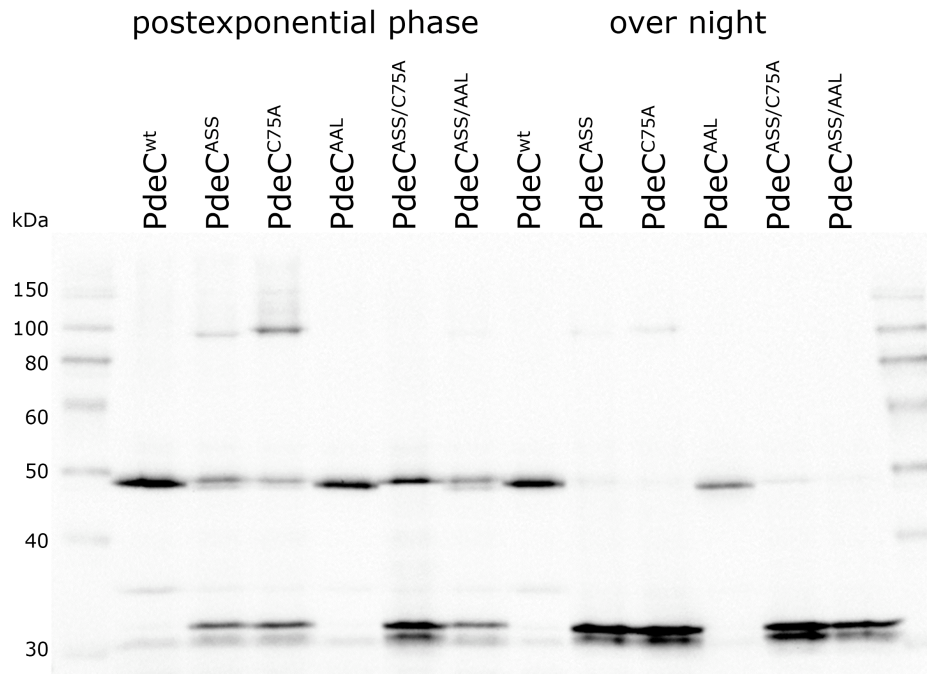

Figure S5. Single cysteine variants of PdeC dimerize by forming intermolecular DSBs. Derivatives of strain W3110 expressing PdeC variants with the indicated mutations from pCAB18-derived plasmids were grown in LB medium at 28ºC and samples were taken at OD_{578} of 2.8 and from overnight cultures. PdeC variants were detected by immunoblotting. Note that dimers are not formed by the PdeC variant that lacks *both* cysteins in the periplasmic domain. Moreover, in overnight cultures of strains expressing $PdeC^{ASS}$ and $PdeC^{C75A}$, the 33 kDa fragment accumulates at the expense of the dimers, indicating that the intermolecular DSB is resolved, which allows the degradation to the 33 kDa fragment.

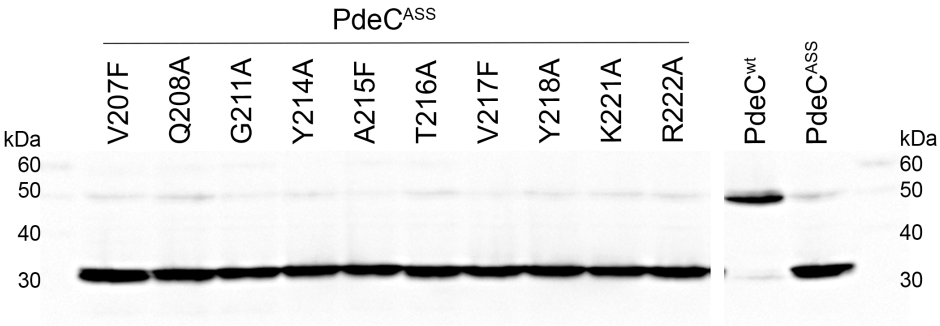

Figure S6. Point mutations between amino acids 207 and 222 in the CSS domain do not affect proteolysis of $PdeC^{ASS}$. Single amino acid exchanges in the periplasmic region of $PdeC^{ASS}$, where cleavage to the 33 kDa fragment occurs, do not affect proteolysis. The 33 kDa fragment obtained from $PdeC^{ASS}$ is approximately 2 kDa larger than the TM2+EAL fragment (see Fig. 5A), which places the putative cleavage site that leads to the formation of the 33 kDa fragment approximately between amino acids 211 and 222. Single amino acids exchanges in and around this region were introduced into $PdeC^{ASS}$ and the gene products were visualized by immunoblot analysis of overnight samples grown in LB at 28ºC.

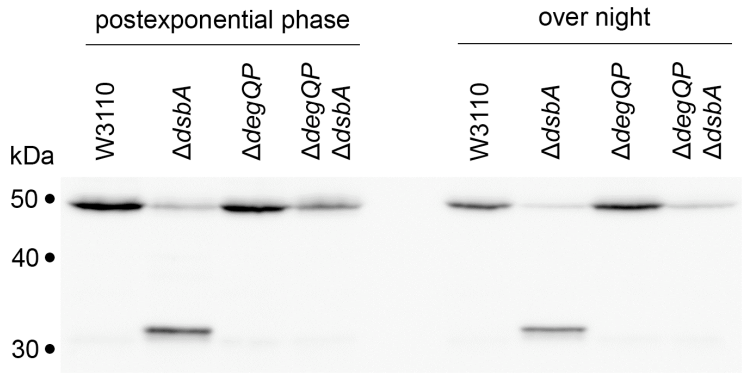

Figure S7. The PdeC^{wt}-derived 33 kDa processed fragment in a *dsbA* mutant is generated by DegP/DegQ. Derivatives of strain W3110 carrying mutations in *dsbA*, *degP* and *degQ* as indicated and expressing PdeC^{wt} from pCAB18-derived plasmids were grown in LB medium at 28°C and samples were taken at OD_{578} of 3.0 (postexponential phase) and from overnight cultures. PdeC and its 33 kDa degradation product were detected by immunoblotting (using His6 tag antibodies).

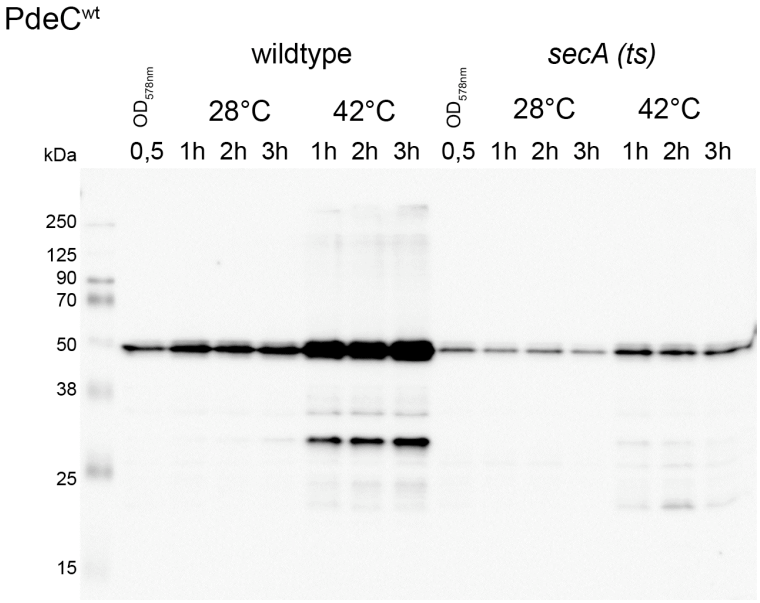

Figure S8. TM1 of PdeC is not a cleavable signal sequence. *E. coli* K-12 strain MC4100 and a temperature-sensitive *secA* mutant derivative expressing PdeC^{wt} from the low copy number plasmic pCAB18 were grown in LB at 28°C to an OD_{578} of 0.5, where the cultures were splitted with incubation continued at 28°C and 42°C. PdeC was detected by immunoblotting (with His6 tag antibodies) in samples taken after splitting of the cultures as indicated. Note that in the *secA^{ts}* mutant, PdeC did not accumulate as a larger fragment after shift to 42°C as observed for exported proteins with cleaved signal sequences (Bassford et al., 1991; Oliver and Beckwith, 1981).

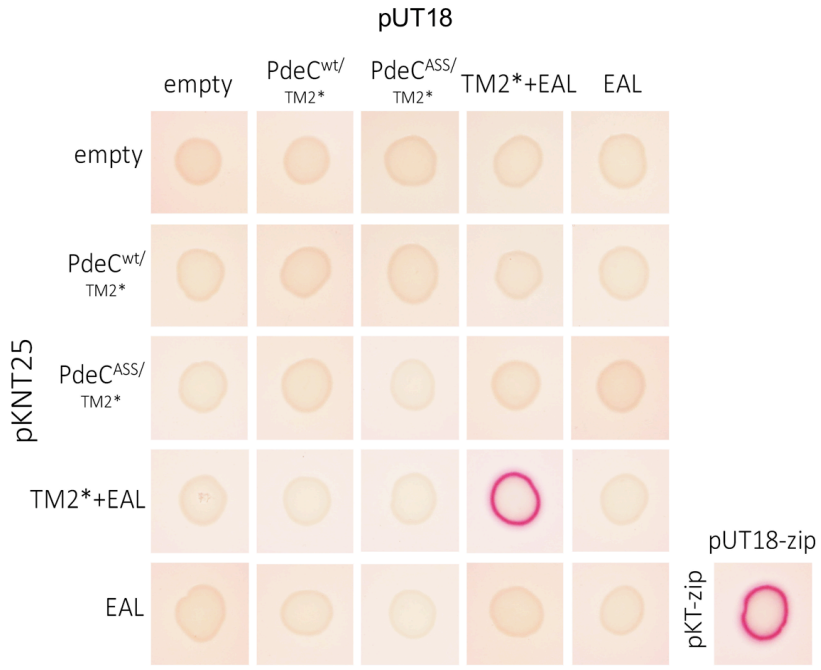

Figure S9. Two-hybrid interaction assays with PdeC derivatives in which TM2 was precisely replaced by the second transmembrane segment of LacY (TM2*). Protein-protein interactions of the indicated PdeC variants carrying TM2* instead of TM2 were tested using the bacterial adenylate cyclase-based two-hybrid system. The experiment was performed as the experiment with the corresponding PdeC constructs with the original TM2 region shown in Fig. 4C, with the exception that the agar plates used here contained 0.1 mM IPTG to also detect possible minor interactions. As a positive control, the leucin zipper part of the yeast GCN4 protein was used.

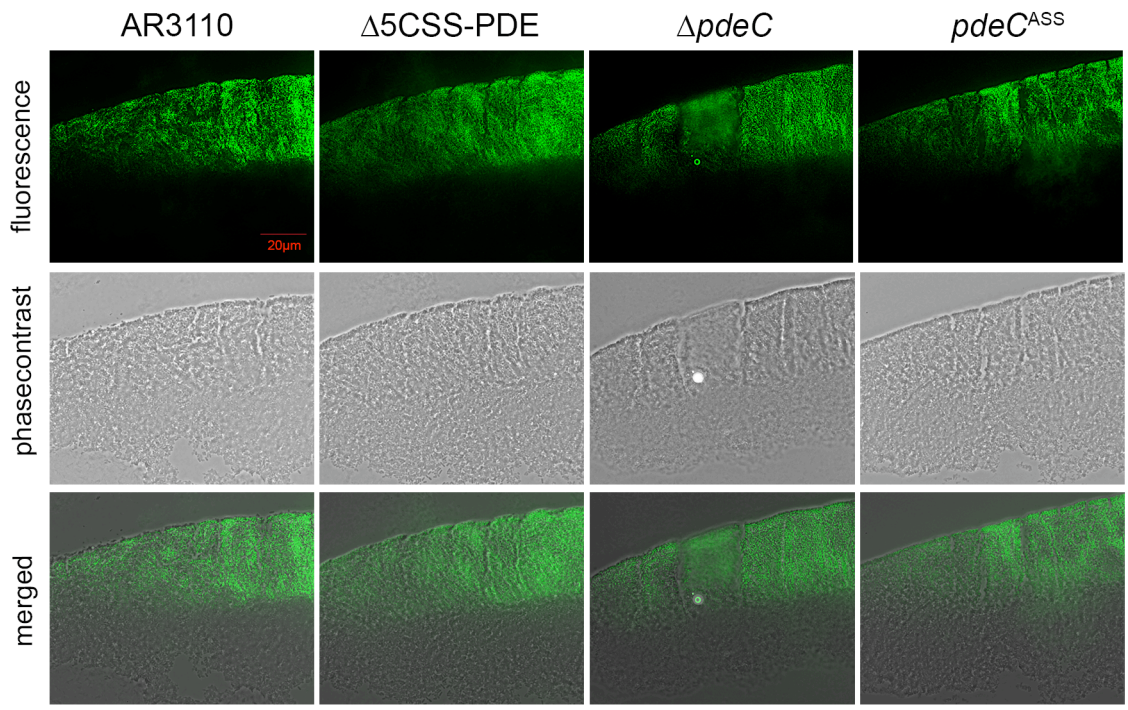

Figure S10. Mutations in *pdeC* or multiple knockouts of genes encoding CSS domain PDEs do not affect matrix distribution or structure in the young outer rim areas of macrocolony biofilms. Macrocolony biofilms of strain AR3110 and the indicated mutant derivatives were grown on salt-free LB containing Thioflavin S (as a matrix-specific fluorescent dye) for 5 d at 28ºC. Vertical fixed cryosections through the outer rim areas of the macrocolonies were visualized by fluorescent microscopy and phasecontrast both separately and in the merged overlay as indicated.

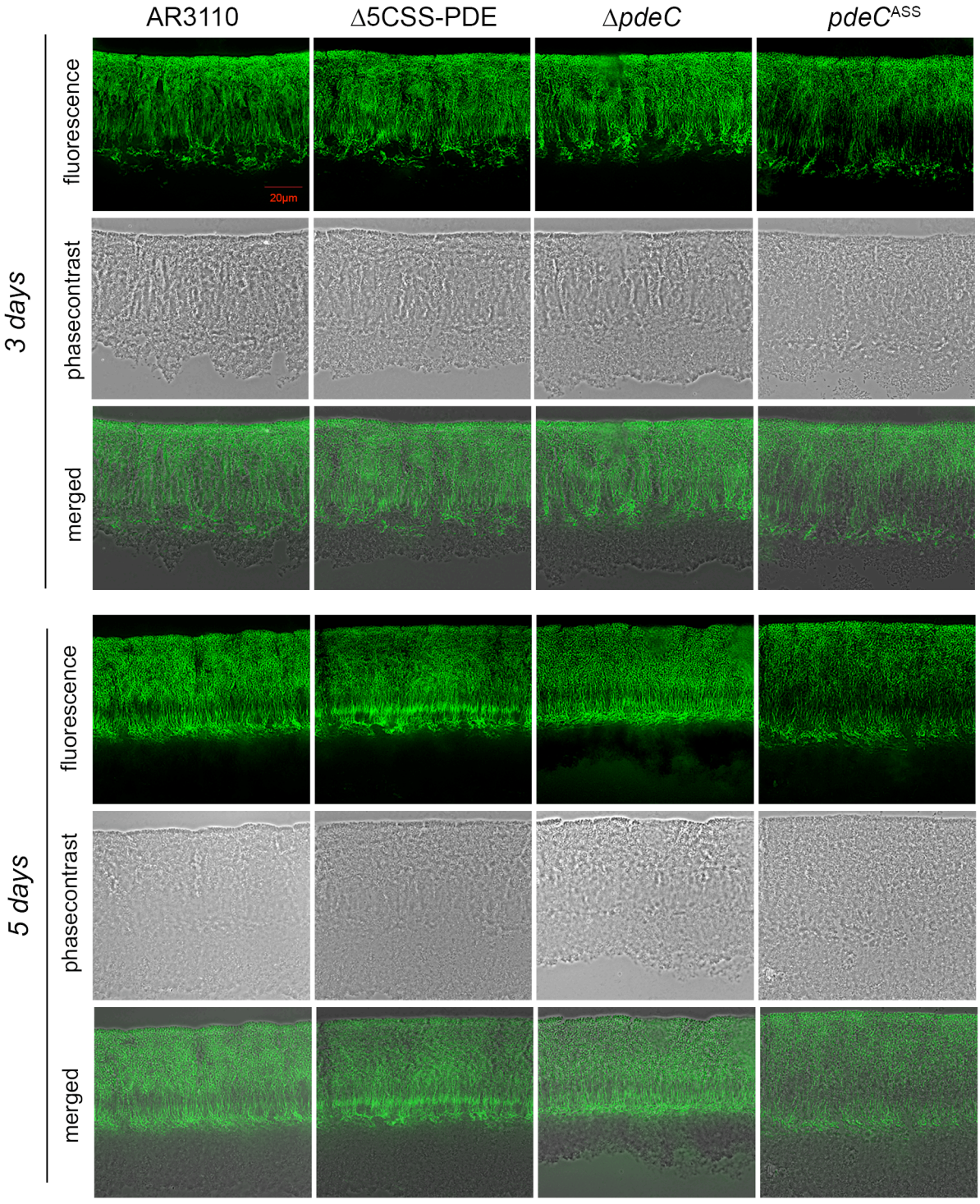

Figure S11. CSS domain PDEs modulate matrix production and architecture in deeper zones of mature macrocolony biofilms. This figure is a complete version of the experiment shown in Fig. 7, showing not only fluorescent images (as in Fig. 7), but also phase contrast and merged fluorescent/phase contrast images.

Supplementary Tables

Table S1. Oligonucleotides used in the present study.

I. Primers used for generating full ORF deletion/insertion mutants by one-step inactivation (OSI):

OSI *dsbA* H1P1	5'- GAGTAGATCatgAAAAAGATTTGGCTGGCGCTGGCTGGTTTGTTTTAG CGTGTAGGCTGGAGCTGCTTC -3'
OSI *dsbA* H2P4	5'- GAATATTCACGGGCTTTATGTAATTTACATTGAAttaTTTTTTCTCAT TCCGGGGATCCGTCGACC -3'
OSI *dsbB* H1P1	5'- GGTTTAAACTGCGCACTCTATGCATATTGCAGGGAAATGATTatgTT GCGGTGTAGGCTGGAGCTGCTTC -3'
OSI *dsbB* H2P4	5'- GCGGCAGGAAAAAAGCGCTCCCGCAGGAGCGCCGAATGGAttaGCGA CCATTCCGGGGATCCGTCGACC -3'
OSI *dsbC* H1P1	5'- CACCCGCGGGCGTGATGTCTGAAAAGAACGGGAAGATTTatgAAGA AAGGGTGTAGGCTGGAGCTGCTTC -3'
OSI *dsbC* H2P4	5'- GACTTCACGGCGACGAAGTTGTATCTGTTGTTTCACGCGAAttaTTTAC CATTCCGGGGATCCGTCGACC -3'
OSI *dsbD* H1P1	5'- CACGGAGACACAGATTACCTCTCatgGCTCAACGCATCTTTACGCTGA TCGTGTAGGCTGGAGCTGCTTC -3'
OSI *dsbD* H2P4	5'- CACGTTGCACGCTATTTTCCTCCGTCTTTCCCACTGCAAGTGTCGTtca ATTCCGGGGATCCGTCGACC -3'
OSI *dsbG* H1P1	5'- CAGTGATAGGTTatgCCTTTTACTCGACTTTTGCACTGACTGAAAAG GACGTGTAGGCTGGAGCTGCTTC -3'
OSI *dsbG* H2P4	5'- CTTttaTTTATTCCCCATAATGATATTAAGCGTTTTCTGATCGGGCAAC CATTCCGGGGATCCGTCGACC -3'
OSI *degP* H1P1	5'- GAAGAACACAGCAATTTTGCGTTATCTGTTAATCGAGACTGAAATAC atgGTGTAGGCTGGAGCTGCTTC -3'
OSI *degP* H2P4	5'- GTAAGGAGAACCCCTTCCCGTTTTCAGGAAGGGGTTGAGGGAGAttaC TGATTCCGGGGATCCGTCGACC -3'
OSI *degQ* H1P1	5'- CTGTTTTGAATCTCTTTTCTTATCATTCAGGTACGAGAGCAGGAATA atgGTGTAGGCTGGAGCTGCTTC -3'
OSI *degQ* H2P4	5'- CGGACATCACACGTAAGCCTGATGCCCGGTTTACGACAttaACGCATC AGATTCCGGGGATCCGTCGACC -3'
OSI *cya* H1P1	5'-GATGTTGGCGGAATCACAGTCATGACGGGTAGCAAATCAGGCGATA CGTCGTGTAGGCTGGAGCTGCTTC-3'
OSI *cya* H2P4	5'-CCGCTAAGATTGCATGCCGGATAAGCCTCGCTTTCCGGCACGTTCAC ATTCCGGGGATCCGTCGACC-3'
OSI *lacI-A* H1P1	5`-TACGTTGACACCATCGAATGGCGCAAAACCTTTCGTGTAGGCT GGAGCTGCTTCG-3`
OSI *lacI-A* H2P4	5`-CCCTGCGTTTTGCACCAGTACGTTTTCCGCAGCTGTGATTCCGG GGATCCGTCGACC-3`

II. Primers used to construct the chromosomal pdeC^{ASS} allele by two-step-mutagenesis:

pdeC^{ASS} H1P1 pKD45	5'-CAGGGTTTTCACCTTGCAATGGCCGGGTATAAACAGGCAGGAAATTGAT AGCGCATCGTGGCCGGATCTTGC-3'
pdeC^{ASS} H2P2 pKD45	5'CAGGCTGTGCGCCAGCTCGATGATGTGTTCCGCAATCAAATGACTGG TTTTGCGGATAACAGAAAGGCCGGG-3'

III. Primers used for constructing pCAB18 derivatives that express PdeC variants or fragments with C-terminal His6-tags[1]:

pdeC fwd EcoRI	5'- GAC**GAATTC**ATGAGTCATCGTGCACGACAC -3'
pdeC rev HindIII	5'- GAC**AAGCTT**TTATTGCCCGCGCGTTAACTC -3'
pdeC rev 6HIS HindIII	5'-GAC**AAGCTT**TTAGTGATGGTGATGGTGATGTT GCCCGCGCGTTAACTC -3'
pdeC fwd 6HIS TM2+EAL	5'- GAC**GAATTC**ATGCATCACCATCACCATCACTCATATC AACGTCTTATAACCCATTTTTAT -3'
pdeC fwd 6HIS EAL	5'- GAC**GAATTC**ATGAGTCATCACCATCACCATCACCCGCCG CGTATTCGACAAAACTATTTATCTCCC -3'
pdeN rev 6HIS SalI	5'- GAC**GTCGAC**TTAGTGATGGTGATGGTGATGCCACTGCGGCG TATACGG -3'
pdeC/N Fusion fwd	5'- CTATTACTCTGGCTACGTATGCGCCCCGGCA -3'
pdeC/N Fusion rev	5'- TGCCGGGGCGCATACGTAGCCAGAGTAATAG -3'

IV. Primers used for introducing mutations into PdeC expressed from pCAB18 derivatives[2]:

pdeC fwd ASS	5'-GATAACCATTTTTTA*GCC*TCATCGCTGATAGC-3'
pdeC rev ASS	5'-GCTATCAGCGATGA*GGC*TAAAAAATGGTTATC-3'
pdeC fwd C75A	5'-GGGGCAAGTT*GCC*ACTCCAGCCCATCAAAAGC-3'
pdeC rev C75A	5'-GCTTTTGATGGGCTGGAGT*GGC*AACTTGCCCC -3'
pdeC fwd AAL	5'-GTATCGGCGCT*GCA*GCGTTGTTACGTTGGC-3'
pdeC rev AAL	5'-GCCAACGTAACAACGC*TGC*AGCGCCGATAC-3'
pdeC fwd V207F	5'-GGATTTAACC*TTC*CAAAGAAATGGCTATTTATATG-3'
pdeC rev V207F	5'-CATATAAATAGCCATTTCTTTG*GAA*GGTTAAATCC-3'
pdeC fwd Q208F	5'-GGATTTAACCGTA*GC*AAGAAATGGCTATTTATATG-3'
pdeC rev Q208F	5'-CATATAAATAGCCATTTCTT*GC*TACGGTTAAATCC-3'
pdeC fwd G211A	5'-GGATTTAACCGTACAAAGAAATG*C*CTATTTATATG-3'
pdeC rev G211A	5'-CATATAAATAG*G*CATTTCTTTGTACGGTTAAATCC-3'
pdeC fwd Y214A	5'-GCTATTTA*GC*TGCGACAGTTTATTCGACAAAACG-3'
pdeC rev Y214A	5'-CGTTTTGTCGAATAAACTGTCGCA*GC*TAAATAGC-3'
pdeC fwd A215F	5'-GCTATTTATAT*TTC*ACAGTTTATTCGACAAAACG-3'
pdeC rev A215F	5'-CGTTTTGTCGAATAAACTGT*GAA*ATATAAATAGC-3'
pdeC fwd T216A	5'-GCTATTTATATGCG*GC*AGTTTATTCGACAAAACG-3'
pdeC rev T216A	5'-CGTTTTGTCGAATAAACT*GC*CGCATATAAATAGC-3'
pdeC fwd V217F	5'-GCTATTTATATGCGACAT*T*TTATTCGACAAAACG-3'
pdeC rev V217F	5'-CGTTTTGTCGAATAAA*A*TGTCGCATATAAATAGC-3'
pdeC fwd Y218A	5'-GCTATTTATATGCGACAGTT*GC*TTCGACAAAACG-3'
pdeC rev Y218A	5'-CGTTTTGTCGAA*GC*AACTGTCGCATATAAATAGC-3'
pdeC fwd K221A	5'-GTTTATTCGACA*GC*ACGCCCAATTGCAGCCATTG-3'
pdeC rev K221A	5'-CAATGGCTGCAATTGGGCGT*GC*TGTCGAATAAAC-3'
pdeC fwd R222A	5'-GTTTATTCGACAAAA*GC*CCCAATTGCAGCCATTG-3'
pdeC rev R222A	5'-CAATGGCTGCAATTGGG*GC*TTTTGTCGAATAAAC-3'

V. Primers used for constructing bacterial two-hybrid plasmids on the basis of the pKNT25 and pUT18 vectors (Karimova et al., 1998) that express PdeC or PdeC fragments fused to the two separate CyaA domains T25 and T18[3]:

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[1] Restriction sites are indicated by **boldface**.

[2] Mutations introduced are indicated by *italics*.

a. External cloning primers:

pdeC^{wt}-XbaI-for	5`-CCG**TCTAGA**GAGTCATCGTGCACGACACC-3`
pdeC^{ASS}-XbaI-for	5`-CCG**TCTAGA**GAGTCATCGTGCACGACACC-3`
pdeC$^{TM2+EAL}$-XbaI-for	5`-CCG**TCTAGA**GCTTATAACCCATTTTTATA-3`
pdeC^{EAL}-XbaI-for	5`-CCG**TCTAGA**GCGTATTCGACAAAACTATT-3`
pdeC-SmaI-rev	5`-TTT**CCCGGG**GTTGCCCGCGCGTTAACTCC-3`

b. Primers introducing TM2*[4]:

TM2*-rev	5`-*GGAATAATAGCGAGAACAGA*GAAATAGCGGCAAAAATAATACCA CGTTGATATGAAGTCGC-3`
TM2*-for	5`-*TCTGTTCTCGCTATTATTCCAACC*GCTGTTTGGTCTGCTTTCTCGTA TTCGACAAAACTAT-3`
pdeC^{TM2*EAL}-XbaI-for	5`-CCG**TCTAGA**G*GGTATTATTTTTGCCGCTATTTCTCTGTTCTCGCTAT TATTCCAACCGCTGTTTGGTCTGCTTTCT*CGTATTCGACAAAACTAT-3`

VI. Synthetic DNA fragments encoding PdeC variants that were directly used for cloning into pCAB18[5] or for replacing larger segments in pdeC on pCAB18[6]:

PdeC$^{\Delta peri}$	5`- GAC**GAATTC**atgAGTCATCGTGCACGACACCAATTACTGGCGTTGCCGGGCATTAT CTTTTTAGTTCTCTTTCCCATCATTCTTTCGCTATGGATTGCCTTCCTTTGGGCAAA ATCAGAAGTGAATAATCAGCTCCGTCTTATAACCCATTTTTATAATCATCTTATTT TTGCGTTGCCCGCCGGTATTTTGGGGAGTCTTGTTCTGCTATTACTCTGGCTACGT ATTCGACAAAACTATTTATCTCCCAAACGTAAATTGCAACGCGCCCTCGAAAAAC ATCAACTTTGTCTTTATTACCAGCCAATAATCGATATCAAAACAGAAAAATGTAT CGGCGCTGAAGCGTTGTTACGTTGGCCTGGTGAGCAGGGGCAAATAATGAATCC GGCAGAGTTTATTCCGCTGGCAGAAAAGGAGGGGATGATAGAACAGATAACTGA TTATGTTATTGATAATGTCTTCCGCGATCTGGGCGATTACCTGGCAACACATGCA GATCGCTATGTTTCTATTAACCTGTCGGCCTCCGATTTTCATACGTCACGGTTGAT AGCGCGAATCAATCAGAAAACAGAGCAATACGCGGTGCGTCCGCAGCAAATTAA ATTTGAAGTGACTGAGCATGCATTTCTTGATGTTGACAAAATGACGCCGATTATT CTGGCTTTCCGCCAGGCAGGTTACGAAGTGGCAATTGATGATTTTGGTATTGGCT ACTCTAACTTGCATAACCTTAAATCATTGAATGTCGATATTTTGAAAATCGACAA ATCGTTTGTTGAAACGCTGACCACCCACAAAACCAGTCATTTGATTGCGGAACAC ATCATCGAGCTGGCGCACAGCCTGGGGTTAAAAACGATCGCTGAAGGCGTCGAA ACTGAGGAGCAGGTTAACTGGCTGCGCAAACGCGGCGTGCGCTATTGCCAGGGA TGGTTCTTTGCGAAGGCGATGCCGCCGCAGGTGTTTATGCAATGGATGGAGCAAT TACCCGCGCGGGAGTTAACGCGCGGGCAATAA**AAGCTT**GTC-3´
PdeC TM1*/TM2*	5'- **GAATTC**atgAGTCATCGTGCACGACACAACTTTTGGATGTTCGGTTTATTCTTTTTC TTTTACTTTTTTATCATGGGAGCCTACTTCCCGTTTTTCCCGATTTGGCTAAAATC AGAAGTGAATAATCAGCTCCGAACCTTTGCTCAACTGGCACTGGATAAATCCGA GCTGGTCATTCGCCAGGCAGATTTAGTGAGCGATGCAGCTGAACGCTATCAGGG GCAAGTTTGCACTCCAGCCCATCAAAAGCGAATGTTGAATATTATTCGTGGCTAT CTTTATATTAATGAATTGATCTATGCCCGTGATAACCATTTTTTATGCTCATCGCT GATAGCGCCTGTAAACGGCTATACGATTGCAC**CGGCCG**ATTATAAGCGTGAACC TAACGTTTCTATCTATTATTACCGCGATACGCCTTTTTTCTCTGGCTATAAAATGA

[3] Restriction sites are indicated by **boldface.**

[4] Sequence parts encoding TM2* are indicated by *italics*; sequences complementary on TM2*-rev and TM2*-for are *underlined*; sequences complementary on TM2*-rev and *pdeC*^{TM2*EAL}-XbaI-for are shown in blue.

[5] Restriction sites are indicated by **boldface.**

[6] Restriction sites are indicated by **boldface**, mutations introduced are indicated by *italics*.

	CCTATATGAGCGGGGAAATTATGTGGCGGTTATCAACCCTCTCTTCTGGAGTGAA GTGATGTCTGATGACCCGACATTGCAATGGGGTGTGTATGATACGGTGACGAAA ACCTTTTTCTCGTTAAGCAAAGAGGCCTCGGCAGCAACGTTTTCTCCGCTGATTC ATTTGAAGGATTTAACCGTACAAAGAAATGGCTATTTATATGCGACAGTTTATTC GACAAAACGCCCAATTGCAGCCATTGTTGCGACTTCATATCAACGTGGTATTATT TTTGCCGCTATTTCTCTGTTCTCGCTATTATTCCAACCGCTGTTTGGTCTGCTTTCT CGTATTCGACAAAACTATTTATCTCCCAAACGTAAATTGCAACGCGCCCTCGAAA AACATCAACTTTGTCTTTATTACCAGCCAATAATC **GATATC**-3`
PdeC$^{EAL\Delta C}$	5`- AACGGCTATACGATTGCAC**CGGCCG**ATTATAAGCGTGAACCTAACGTTTCTATC TATTATTACCGCGATACGCCTTTTTTCTCTGGCTATAAAATGACCTATATGCAGCG GGGAAATTATGTGGCGGTTATCAACCCTCTCTTCTGGAGTGAAGTGATGTCTGAT GACCCGACATTGCAATGGGGTGTGTATGATACGGTGACGAAAACCTTTTTCTCGT TAAGCAAAGAGGCCTCGGCAGCAACGTTTTCTCCGCTGATTCATTTGAAGGATTT AACCGTACAAAGAAATGGCTATTTATATGCGACAGTTTATTCGACAAAACGCCC AATTGCAGCCATTGTTGCGACTTCATATCAACGTCTTATAACCCATTTTTATAATC ATCTTATTTTTGCGTTGCCCGCCGGTATTTTGGGGAGTCTTGTTCTGCTATTACTC TGGCTACGTATTCGACAAAACTATTTATCTCCCAAACGTAAATTGCAACGCGCCC TCGAAAAACATCAACTT*A*GTCTTTATTACCAGCCAATAATCGATATCAAAACAGA AAAA*A*GTATCGGCGCTGAAGCGTTGTTACGTTGGCCTGGTGAGCAGGGGCAAAT AATGAATCCGGCAGAGTTTATTCCGCTGGCAGAAAAGGAGGGGATGATAGAACA GATAACTGATTATGTTATTGATAATGTCTTCCGCGATCTGGGCGATTACCTGGCA ACACATGCAGATCGCTATGTTTCTATTAACCTGTCGGCCTCCGATTTTCATACGTC ACGGTTGATAGCGCGAATCAATCAGAAAACAGAGCAATACGCGGTGCGTCCGCA GCAAATTAAATTTGAAGTGACTGAGCATGCATTTCTTGATGTTGACAAAATGACG CCGATTATTCTGGCTTTCCGCCAGGCAGGTTACGAAGTGGCAATTGATGATTTTG GTATTGGCTACTCTAACTTGCATAACCTTAAATCATTGAATGTCGATATTTTGAA AATCGACAAATCGTTTGTTGAAACGCTGACCACCCACAAAACCAGTCATTTGATT GCGGAACACATCATCGAGCTGGCGCACAGCCTGGGGTTAAAAACGATCGCTGAA GGCGTCGAAACTGAGGAGCAGGTTAACTGGCTGCGCAAACGCGGCGTGCGCTAT *A*GCCAGGGATGGTTCTTTGCGAAGGCGATGCCGCCGCAGGTGTTTATGCAATGGA TGGAGCAATTACCCGCGCGGGAGTTAACGCGCGGGCAACATCACCATCACCATC ACtaa**AAGCTT**GTC-3`

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