nature microbiology

Supplementary information

https://doi.org/10.1038/s41564-025-02027-2

Cyclic-di-AMP modulates cellular turgor in response to defects in bacterial cell wall synthesis

In the format provided by the authors and unedited

Strain Construction:

The following oligonucleotide primers were used to make the indicated deletions:

(cdaAR)::tet (oAB76/231 & oAB232/79); *cdaA::tet (oAB76/77 & oAB195/196); disA::tet* (oAB85/86 & oAB87/88); *cdaS::tet* (oAB89/90 & oAB91/92); *gdpP::tet* (oAB200/722 & oAB202/203); *pgpH::tet* (oAB248/249 & oAB721/251); *ponA::kan* (oAB16/17 & oAB18/19). Antibiotic cassettes were amplified with oAB27/28.

BAB1178 - yhdG::Phy-spank-cdaS(L44F) (spc)
B. subtilis PY79 [yhdG::cat] was transformed with a 2-piece isothermal assembly using a PCR product amplified from pAB266 [yhdG-Phy-spank-cdaS (spc) (amp)] using oAB315 & oAB521, and a PCR product amplified from pAB266 [yhdG-Phy-spank-cdaS (spc) (amp)] using oAB316 & oAB531.

BAB1306 - yhdG::Pspank-cdaS(L44F) (spc)
B. subtilis PY79 [yhdG::cat] was transformed with a 2-piece isothermal assembly using a PCR product amplified from gAB280 [yhdG::Pspank-cdaA-His6 (spc)] using oAB548 & oAB550, and a PCR product amplified from gAB226 [yhdG::Phy-spank-cdaS(L44F) (spc)] using oAB518 & oAB549.

Plasmid Construction:

pAB45 [yhdG::Pspank-cdaAR (spc)(amp)]
pAB45 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB83 and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB46 [yhdG::Pspank-cdaAR-ΔID (spc)(amp)]
pAB46 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB84 and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB124 [ycgO::Pveg-kimA-optRBS-lacZ (erm) (amp)]
pAB124 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB224 & oAB217, a PCR product amplified from Bs168 gDNAusing oAB216 & oAB218, and pYB64 [ycgO::lacZ] digested with BamHI & EcoRI.

pAB126 [yhdG::Pspank-cdaAR-ΔID-His (spc) (amp)]
pAB126 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB223 and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB127 [yhdG::Pspank-cdaAR-His (spc) (amp)]
pAB127 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB222 and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB135 [yhdG::Pspank-cdaARΔID-ponAIDR (spc) (amp)]
pAB135 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB270, a PCR product amplified from PY79 gDNA using oAB258 & oAB259, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB136 [yhdG::Pspank-cdaARΔID-rsgIIDR (spc) (amp)]
pAB136 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB271, a PCR product amplified from PY79 gDNA using oAB260 & oAB261, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB137 [yhdG::Pspank-cdaARΔID-scramble1 (spc) (amp)]
pAB137 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB272, a PCR product amplified from CdaRScr1 GBlock using oAB262 & oAB263, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB144 [miniMAD-cdaR-His6 (erm) (amp)]
pAB144 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB273 & oAB274, a PCR product amplified from PY79 gDNA using oAB275 & oAB276, and pMADmini digested with BamHI & HindIII.

pAB146 [yhdG::PxylA-cdaAR (spc) (amp)]
pAB146 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB278 & oAB279 and pCB107 [yhdG::PxylA (spc)] digested with HindIII and BamHI.

pAB147 [yhdG::PxylA-cdaAR-ΔID(spc) (amp)]
pAB147 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB278 & oAB280 and pCB107 [yhdG::PxylA (spc)] digested with HindIII and BamHI.

pAB148 [pminiMAD2-cdaA-D171A (erm) (amp)]
pAB148 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB281 & oAB282, a PCR product amplified from PY79 gDNA using oAB283 & oAB284, and pMADmini digested with EcoRI & HindIII.

pAB150 [yhdG::Pspank-cdaARΔID-BcereusCdaRIDR (spc) (amp)]
pAB150 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB323, a PCR product amplified from B. cereus CdaR GBlock using oAB308 & oAB309, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB151 [yhdG::Pspank-cdaARΔID-EfaecalisCdaRIDR (spc) (amp)]
pAB151 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB324, a PCR product amplified from E. faecalis CdaR GBlock using oAB310 & oAB311, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB162 [yhdG::Pspank-cdaA (spc) (amp)]
pAB162 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB307 and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB175 [yhdG::Pspank-cdaARΔID-scramble2 (spc) (amp)]
pAB175 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB340, a PCR product amplified from CdaR Scr2 GBlock using oAB338 & oAB339, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB176 [yhdG::Pspank-cdaARΔID-scramble3 (spc) (amp)]
pAB176 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB341, a PCR product amplified from CdaR Scr3 GBlock using oAB338 & oAB339, and pCB58 [yhdG::Pspank (spc)] digested with HindIII and SpeI.

pAB191 [yhdG::PxylA-cdaAR-His6 (spc) (amp)]

pAB191 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pAB127 [yhdG::Pspank-cdaAR-His (spc) (amp)] using oAB278 & oAB370, and a PCR product amplified from pCB107 [yhdG::PxylA (spc)] using oAB407 & oAB408.

pAB192 [yhdG::PxylA-cdaAR-ΔID-His6 (spc) (amp)]
pAB192 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pAB126 [yhdG::Pspank-cdaAR-ΔID-His (spc) (amp)] using oAB278 & oAB371, and a PCR product amplified from pCB107 [yhdG::PxylA (spc)] using oAB407 & oAB408.

pAB193 [yhdG::PxylA-cdaARΔID-Scr1 (spc) (amp)]
pAB193 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pAB137 [yhdG::Pspank-cdaAR-Scr1 (spc) (amp)] using oAB278 & oAB372, and a PCR product amplified from pCB107 [yhdG::PxylA (spc)] using oAB407 & oAB408.

pAB198 [yhdG::PxylA-cdaARΔID-BcereusIDR (spc) (amp)]
pAB198 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pAB150 [yhdG::Pspank-cdaARΔID-BcereusCdaRIDR (spc) (amp)] using oAB278 & oAB377, and a PCR product amplified from pCB107 [yhdG::PxylA (spc)] using oAB407 & oAB408.

pAB200 [yhdG::Pspank-cdaARΔID-scramble1-His6 (spc) (amp)]
pAB200 was generated in a KLD reaction using a PCR product amplified from pAB137 [yhdG--Pspank-cdaARΔID-scramble1 (spc) (amp)] using oAB429 & oAB406.

pAB201 [yhdG::Pspank-cdaARΔID-scramble2-His6 (spc) (amp)]
pAB201 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from gAB123 [yhdG--Pspank-cdaAR-Sc21 (spc)] using oAB82 & oAB380, and pCB58 [yhdG::Pspank (spc)] digested with HindIII & SpeI.

pAB202 [yhdG::Pspank-cdaARΔID-scramble3-His6 (spc) (amp)]
pAB202 was generated in a KLD reaction using a PCR product amplified from pAB176 [yhdG--Pspank-cdaARΔID-scramble3 (spc) (amp)] using oAB430 & oAB406.

pAB220 [yhdG::Pspank-disA (spc) (amp)]
pAB220 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB427 & oAB428, and pCB58 [yhdG::Pspank (spc)] digested with HindIII & SpeI.

pAB266 [yhdG::Phy-spank-cdaS (spc) (amp)]
pAB266 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB518 & oAB519, and pCB110 [yhdG::Phy-spank (spc)] digested with HindIII & SpeI.

pAB280 [yvbJ::Pspank-ponA (cat) (amp)]
pAB280 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB168 & oAB169, and pCB118 [yvbJ::Pspank (cat)] digested with HindIII & SpeI.

pAB300 [yhdG::Pspank-cdaA-His6 (spc) (amp)]
pAB300 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB82 & oAB582, and pCB58 [yhdG::Pspank (spc)] digested with HindIII & SpeI.

pAB301 [yvbJ::PxylA-cdaR-HA (cat) (amp)]

pAB301 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB586 & oAB584, and pCB130 [yvbJ::PxylA (cat)] digested with HindIII & BamHI.

pAB302 [yvbJ::PxylA-cdaR-ΔID-HA (cat) (amp)]
pAB302 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB586 & oAB585, and pCB130 [yvbJ::PxylA (cat)] digested with HindIII & BamHI.

pAB310 [yhdG::Pspank-cdaA-D171A-His6 (spc) (amp)]
pAB310 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from gAB142 [ycgO::Pspank-ponA ponA::cat cdaA-D171A] using oAB82 & oAB582, and pCB58 [yhdG::Pspank (spc)] digested with HindIII & SpeI..

pAB355 [yhdG::Phy-spank-optRBS-gdpP(cyto) (spec) (amp)]
pAB355 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB744 & oAB743, and pCB101 [yhdG::Phy-spank (kan)] digested with HindIII & SpeI.

pAB429 [yhdG::Pspank-optRBS-gfp (kan) (amp)]
pAB429 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from gAB190 gDNA [sacA::Pveg-optRBS-gfp] using oAB987/988 and pCB59 [yhdG::Pspank (kan)] digested with SpeI & PstI.

optRBS indicates an optimized RBS with the sequence cataaggaggaactact. All plasmids were verified by sanger or nanopore sequencing.

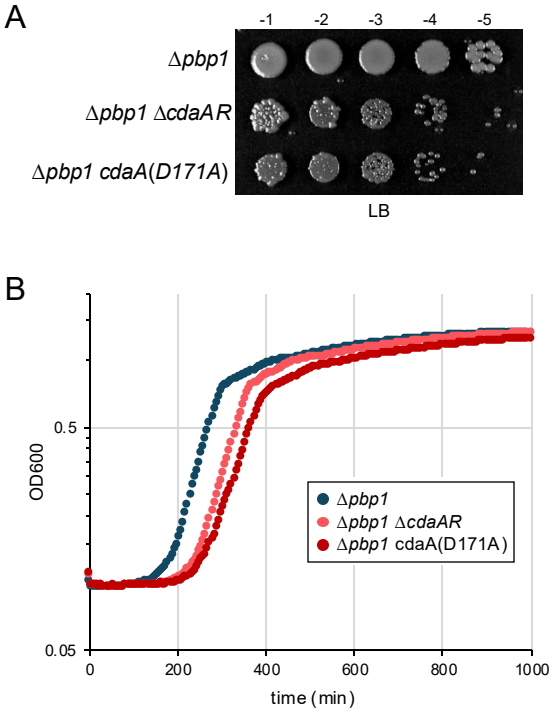

Figure S1. CdaA activity is important in the absence of PBP1. (A) Spot dilutions of the indicated *B. subtilis* strains on LB agar. The catalytic mutant *cdaA*(D171A) mutant has an amino acid substitution at the native *cdaA* locus. **(B)** Representative growth curve of the indicated strains grown in LB at 37°C. Growth curves and spot titers were performed in biological triplicate and a representative experiment is shown.

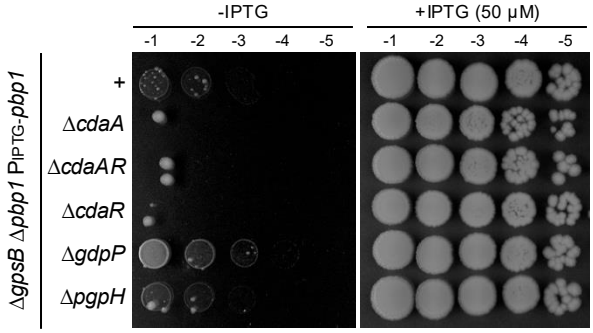

Figure S2. Cells lacking *gpsB* and the indicated cyclase or phosphodiesterase grow similarly to Δ*gpsB*. 10-fold serial dilutions of the indicated strains were spotted on LB agar in the presence or absence of IPTG. The level of PBP1 resulting from induction of P$_{IPTG}$-*pbp1* with 50 µM IPTG is similar to wild-type (30). The Δ*gpsB* mutant grows identically to wild-type on LB (Response reference 10-11).

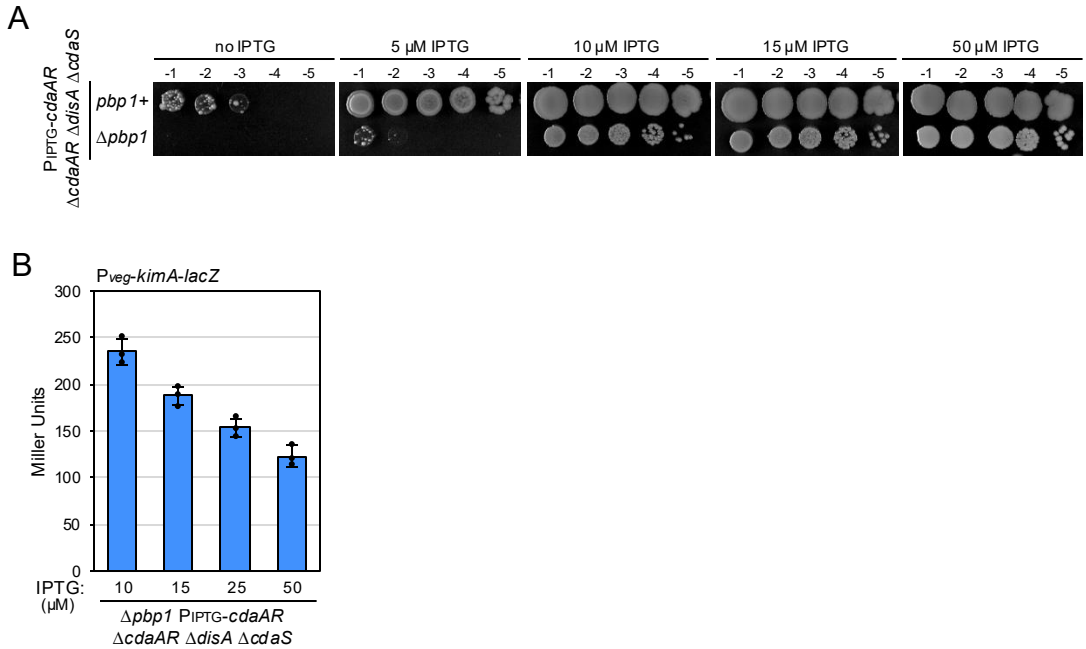

Figure S3. Small changes in c-di-AMP levels can have large phenotypic consequences in cells with impaired PG synthesis. (A) 10-fold serial dilutions of the indicated *B. subtilis* strains were spotted on LB agar supplemented with the indicated concentrations of IPTG. Reducing CdaAR expression from 10 µM to 5 µM IPTG results in loss of viability in cells lacking PBP1. **(B)** Bar graph showing β-galactosidase activity from the *kimA* riboswitch reporter in cells grown in the presence of the indicated IPTG concentrations. The center of error represents the average, and the error bars indicate standard deviation among three biological replicates.

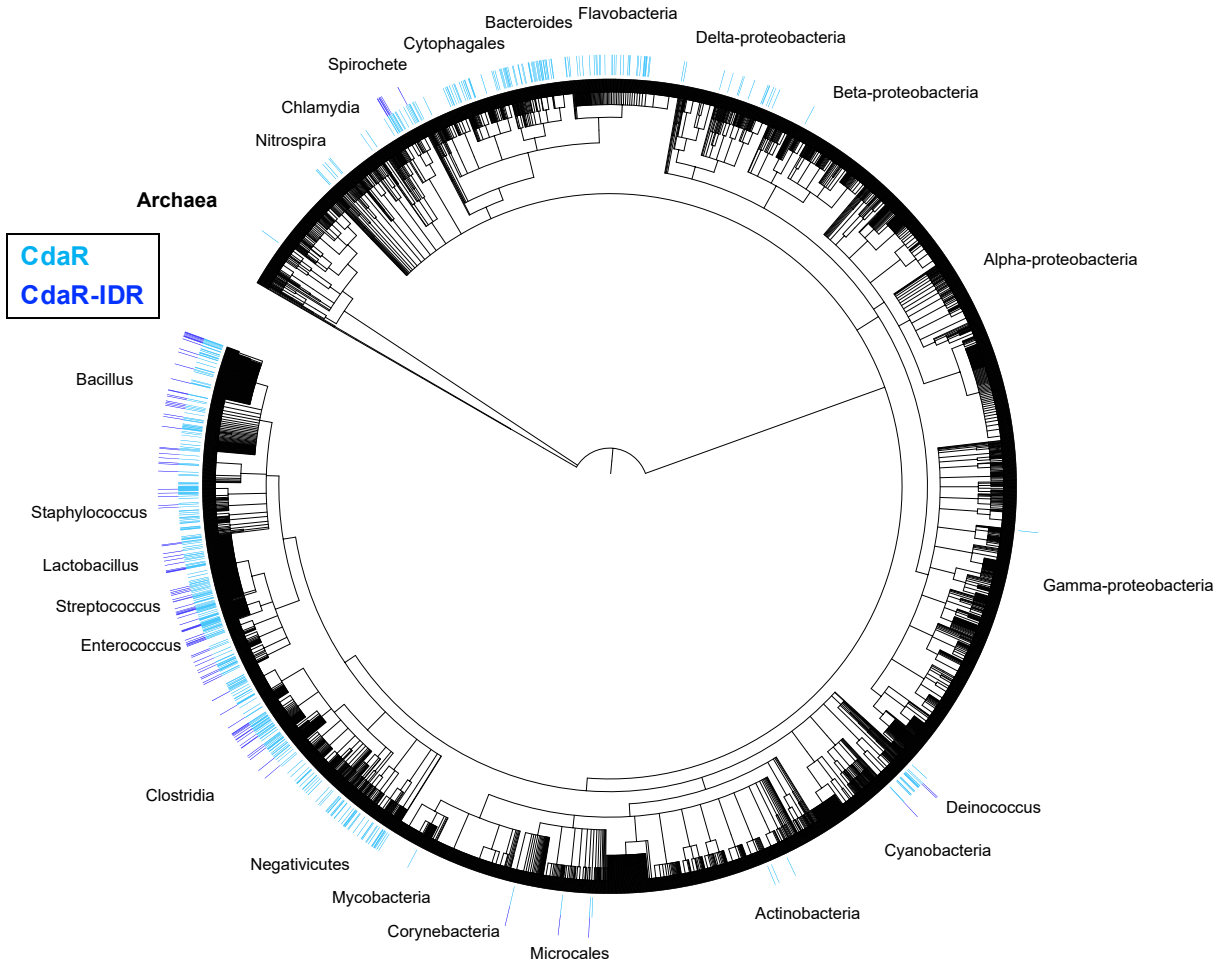

Figure S4. The presence of an IDR on CdaR is broadly conserved. Phylogenetic tree showing distribution of CdaR homologs throughout 5767 bacterial taxa. The amino acid sequence of *B. subtilis* CdaR was used to query the Refseq database and the resulting species containing CdaR homologs are annotated in light blue. Each sequence was analyzed for the presence of >20 amino acids of disorder at their extreme C-terminus using IUPRED (66). The resulting species containing IDRs are highlighted in dark blue.

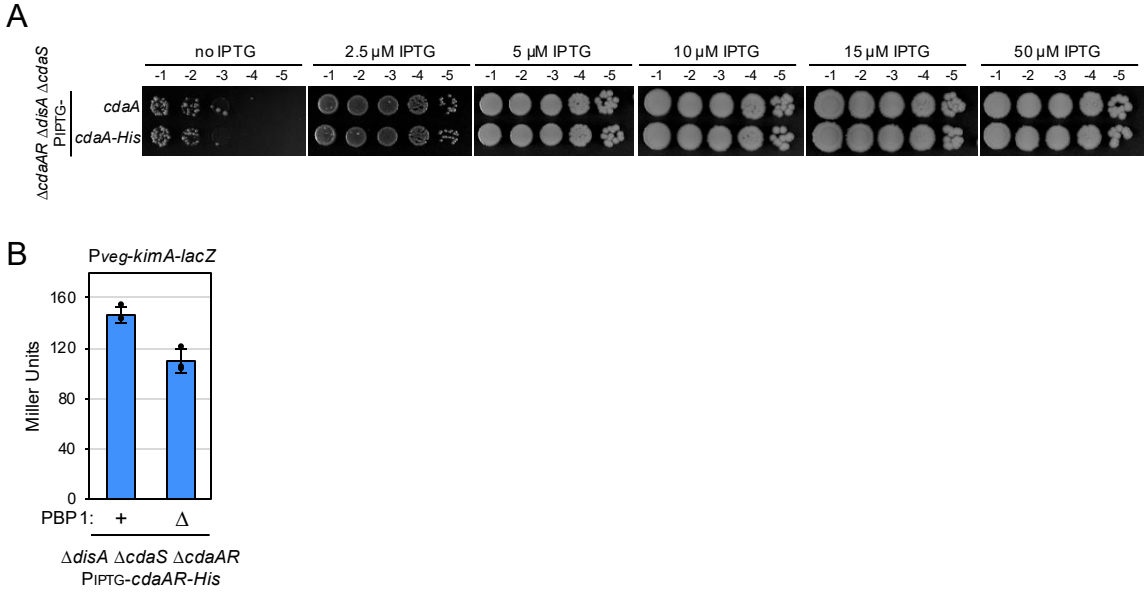

Figure S5. His-tagged alleles of *cdaA* and *cdaR* are functional. (A) 10-fold serial dilutions of the indicated strains were spotted on LB agar containing the indicated concentrations of IPTG. The CdaA-His fusion has a hexa-histidine tag fused to the C-terminus of CdaA. Both constructs support growth to an identical degree indicating that the fusion protein is functional. **(B)** Bar graph showing β-galactosidase activity from the c-di-AMP riboswitch reporter (P*veg-kimA-lacZ*) in the indicated *B. subtilis* strains. The *cdaA-cdaR-His6* operon (*cdaAR-His*) is expressed with 50 µM IPTG in the presence (+) or absence (Δ) of PBP1. The center of error represents the average, and the error bars indicate standard deviation among three biological replicates.

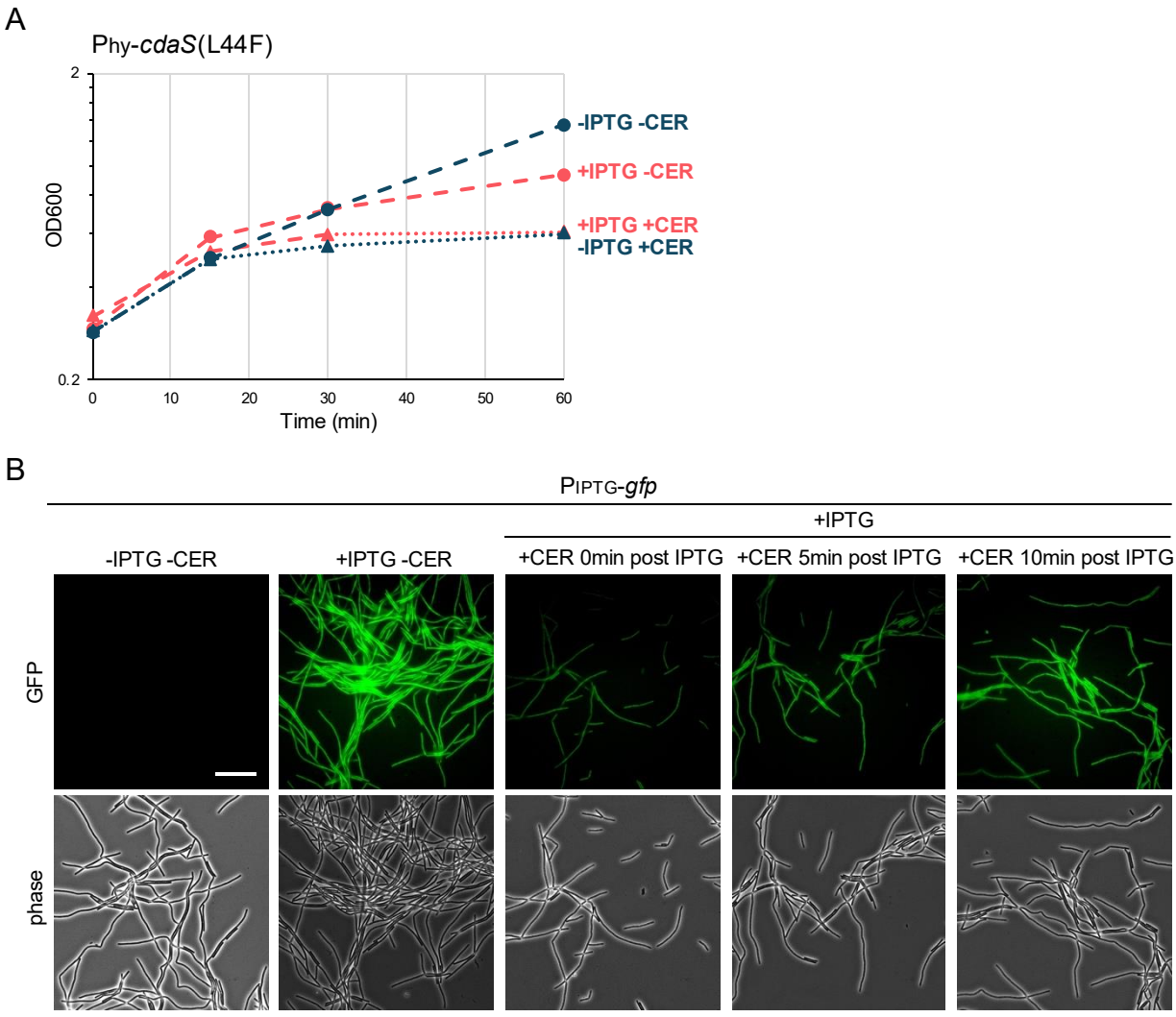

Figure S6. Cerulenin inhibits growth and gene expression. (A) Growth curve of *B. subtilis* cells harboring Phy-*cdaS*(L44F) induced with 500 µM IPTG (red lines) or without inducer (blue lines). Cells were treated with (triangles) or without (circles) 12.5 µg/mL cerulenin. Treatment of cells with cerulenin immediately halts growth independent of IPTG induction, suggesting a reduction in cellular metabolism. **(B)** Representative fluorescence and phase-contrast micrographs of *B. subtilis* cells harboring an IPTG-inducible allele of *gfp* (PIPTG-*gfp*). Cells were induced (+IPTG) with 500 µM IPTG and then cells were treated with 12.5 µg/mL cerulenin 0, 5, or 10 min later. All cells were harvested at 15 min after IPTG addition. Images were acquired ~5 minutes due to cell concentration, mounting, and imaging. Scale bar indicates 5 µm. Cells treated with cerulenin at 0 or 5 min after IPTG addition had diminished GFP expression. The micrographs shown are representative of two biological replicates.

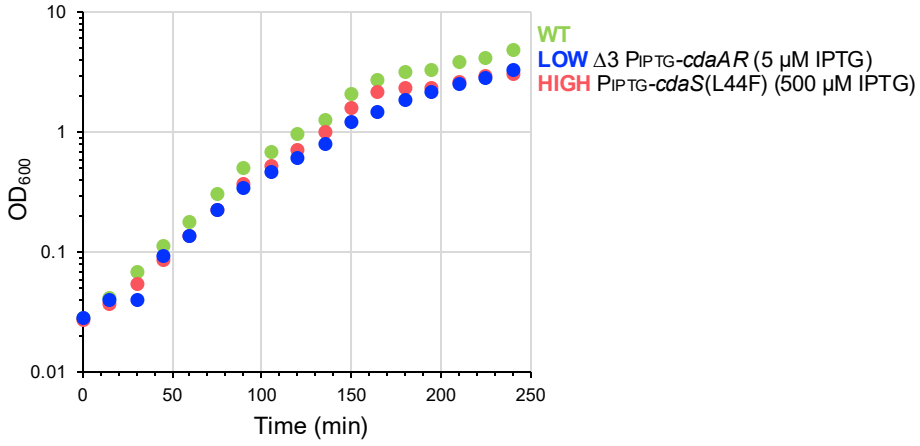

Figure S7. Cells with low, wild-type, or high levels of c-di-AMP grow at similar rates. Growth curves in LB medium at 37 ˚C of wild-type (WT) (green), cells with high c-di-AMP (HIGH) harboring PIPTG-*cdaS*(L44F) grown with 500 μM IPTG (red), and cells with low c-di-AMP (LOW) that lack *disA*, *cdaS*, *cdaAR* and harbor PIPTG-*cdaAR* grown with 5 μM IPTG (blue). The growth curves points are derived from one experiment and are representative of three biological replicates.

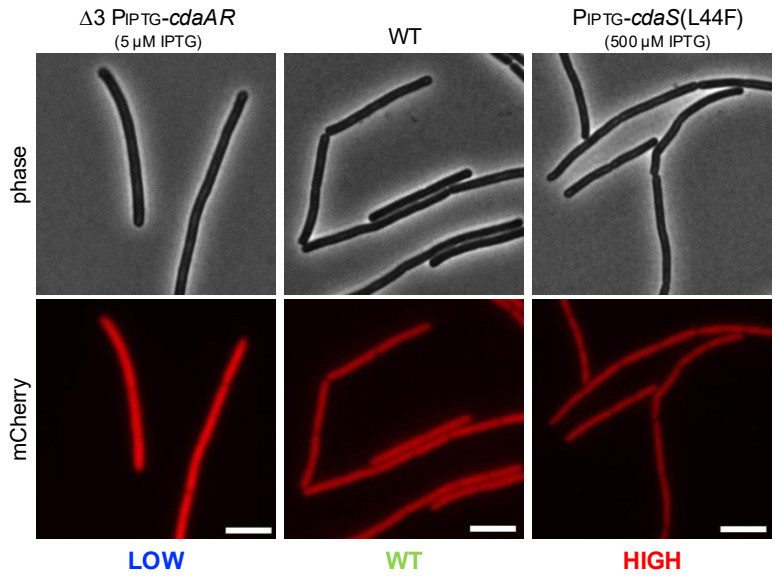

Figure S8. C-di-AMP levels inversely correlate with cell width. Representative fluorescence and phase-contrast images used for width quantification in Figure 5H. Strains were wild-type (WT), cells expressing P_{IPTG}-*cdaS*(L44F) with 500 μM IPTG (HIGH c-di-AMP), and cells lacking *disA*, *cdaS*, *cdaAR* and harboring an IPTG-regulated allele of *cdaAR* with 5 μM IPTG (LOW c-di-AMP). All cells constitutively express cytoplasmic mCherry which was used for width quantification. Scale bars indicate 5 μm.