Supplemental Data

Functional characterization of core components of the *Bacillus subtilis* c-di-GMP signaling pathway

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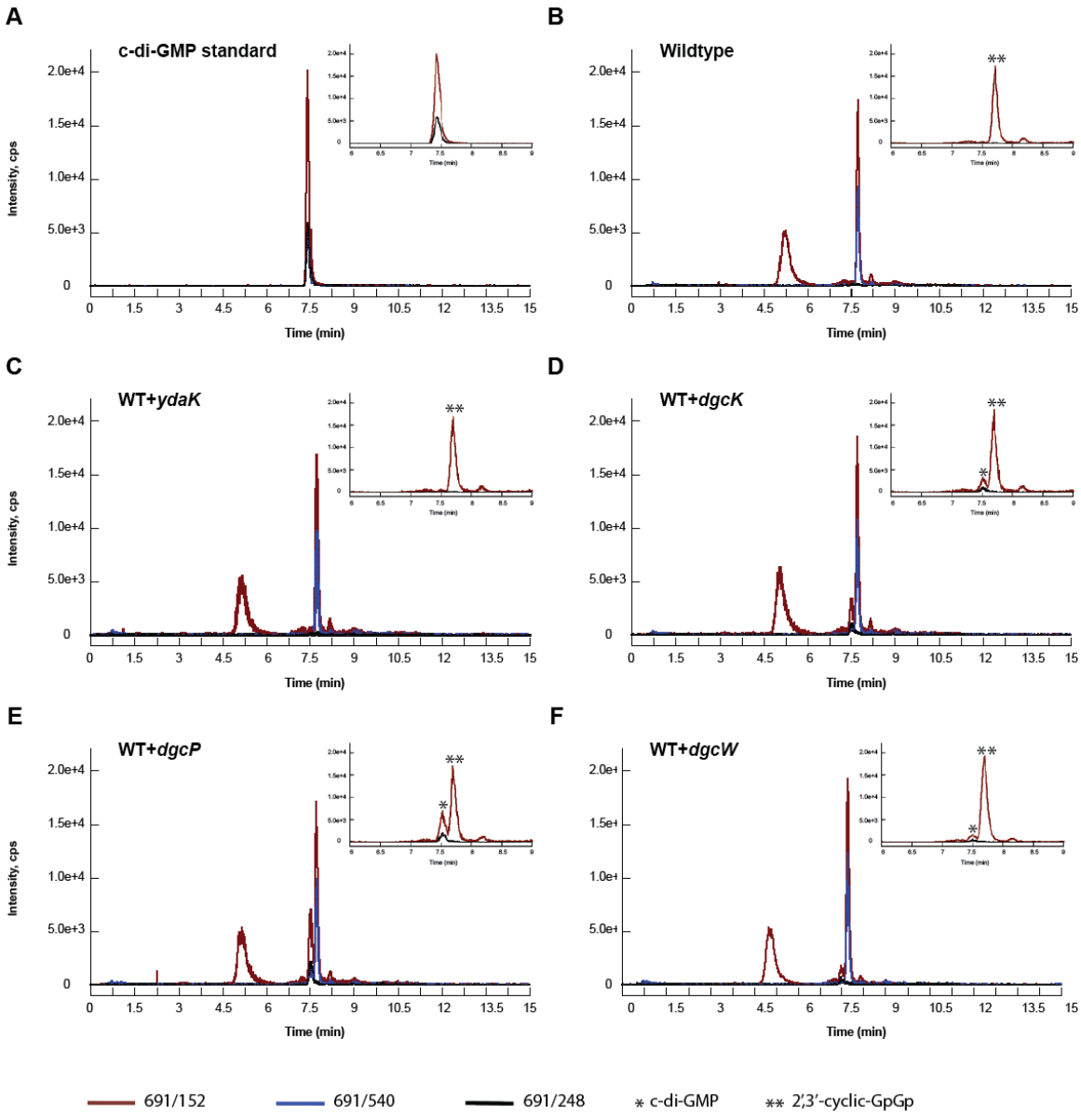


FIG. S1. *In vivo* analysis of c-di-GMP production by putative DGCs in inducible expression strains derived from wild type *B. subtilis*. All signals obtained from MRM transitions *m/z* 691 → 152 (red), *m/z* 691 → 540(blue) and *m/z* 691 → 248 (black) via LC-MS/MS are displayed for (A) c-di-GMP standard and bacterial extracts of (B) wild type *B. subtilis* and strains expressing putative DGCs: (C) YdaK, (D) DgcK (E) DgcP, and (F) DgcW. Transitions *m/z* 691 → 152 (red), *m/z* 691 → 540 (blue) are not unique to c-di-GMP in *B. subtilis* lysates. Only two transitions are shown in insets to indicate relative abundance of the unique identifier (*m/z* 691 → 248 in black) and the most abundant fragmentation product (*m/z* 691 → 152 in red).

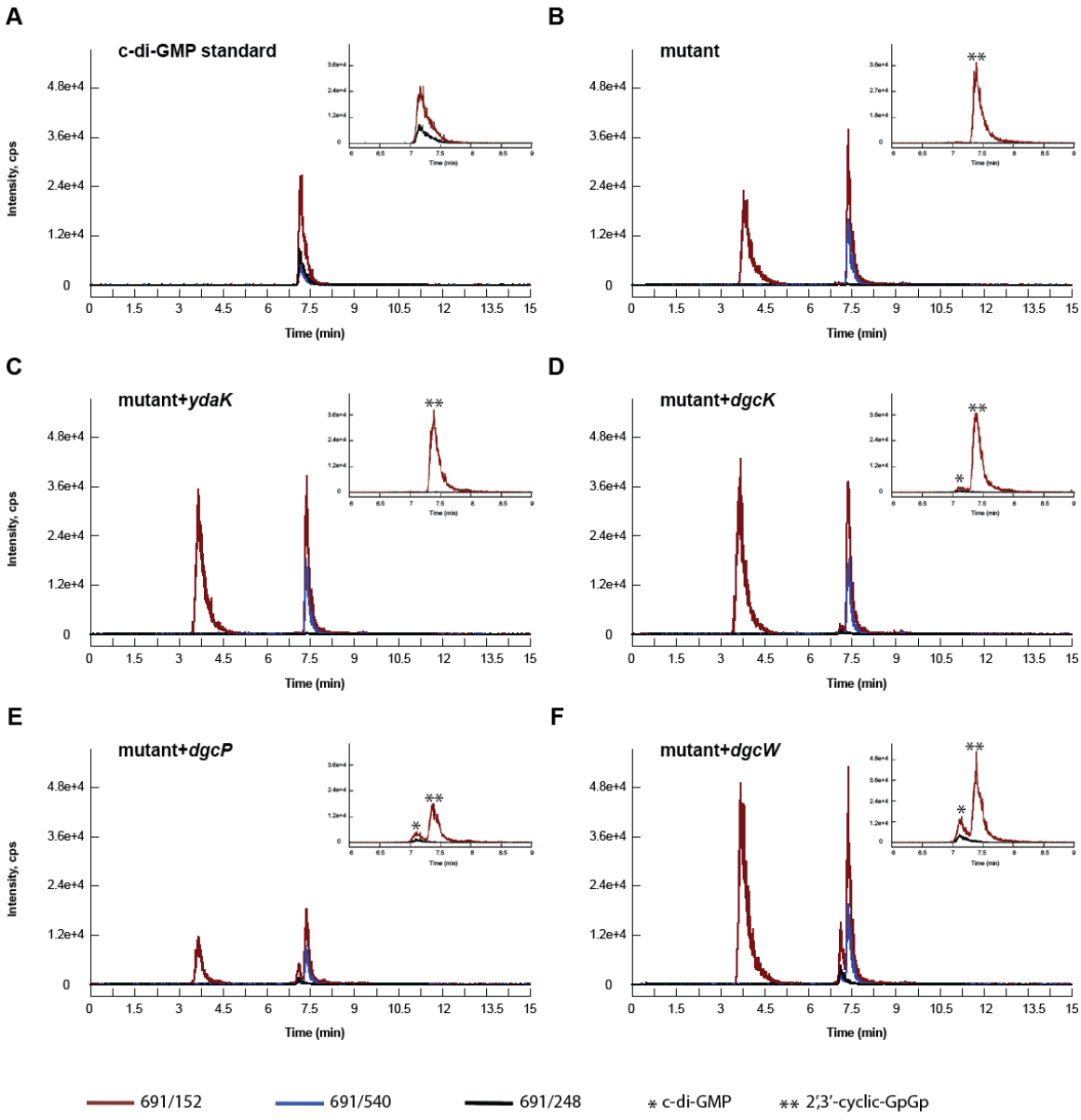


FIG. S2. ***In vivo*** **analysis of c-di-GMP production by putative DGCs in a background lacking all endogenous DGCs.** All signals obtained from MRM transitions *m/z* 691 → 152 (red), *m/z* 691 → 540 (blue) and *m/z* 691 → 248 (black) via LC-MS/MS are displayed for (A) c-di-GMP standard and bacterial extracts of (B) *ΔydaK dgcK dgcW dgcP :: tet B. subtilis* - labeled as mutant - and strains expressing putative DGCs: (C) YdaK, (D) DgcK (E) DgcP, and (F) DgcW. Transitions *m/z* 691 → 152 (red), *m/z* 691 → 540 (blue) are not unique to c-di-GMP in *B. subtilis* lysates. Only two transitions are shown in insets to indicate relative abundance of the unique identifier (*m/z* 691 → 248 in black) and the most abundant fragmentation product (*m/z* 691 → 152 in red).

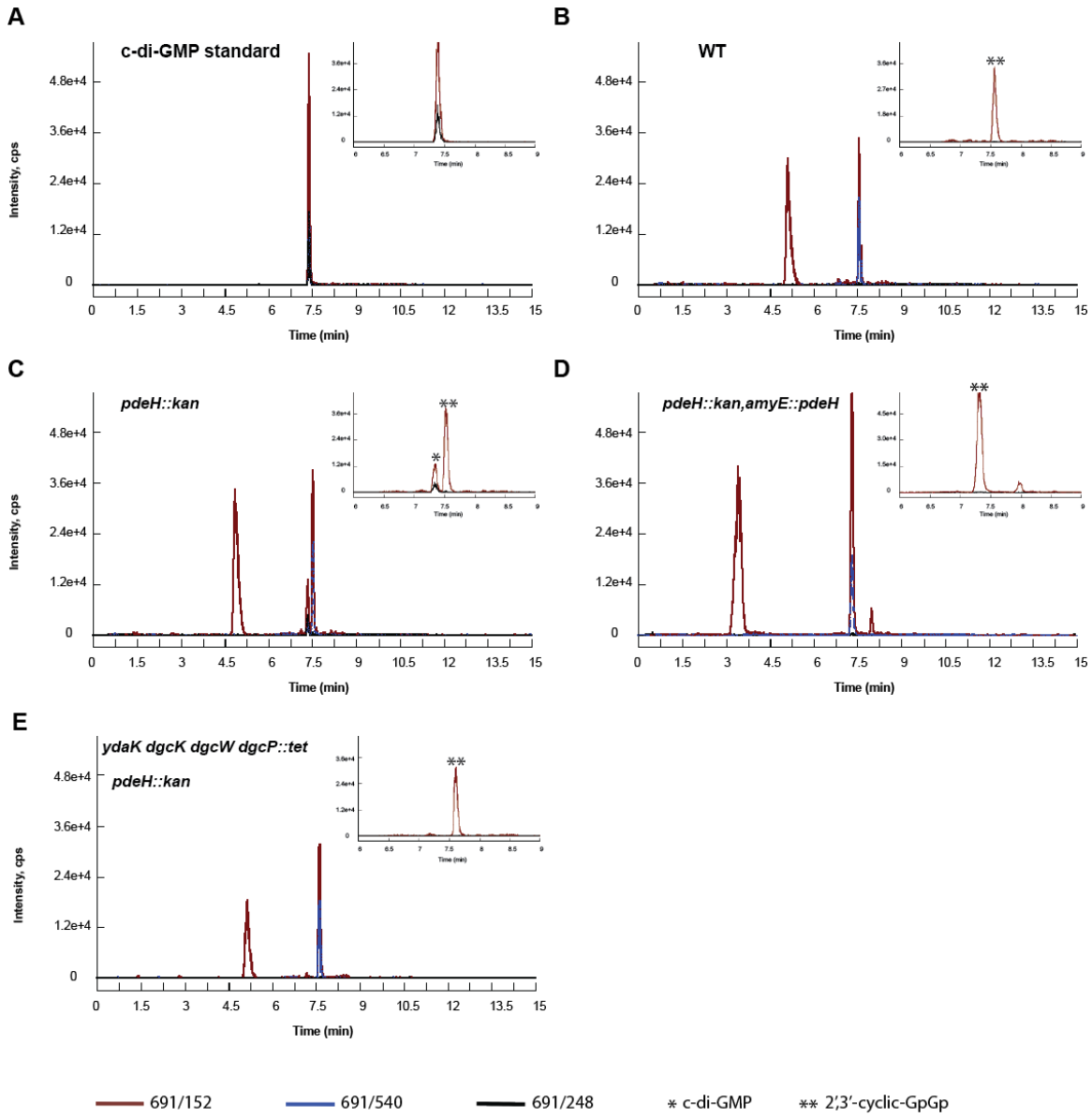


FIG. S3. *In vivo* detection of c-di-GMP in knockout strains of PdeH, a c-di-GMP phosphodiesterase. LC-MS/MS was used to detect c-di-GMP via the MRM transitions *m/z* 691 → 152 (red), *m/z* 691 → 540 (blue) and *m/z* 691 → 248 (black) for c-di-GMP standard (A) and *B. subtilis* extracts from the following strains: (B) wild type;(C) *Δ pdeH::kan* lacking a putative c-di-GMP Phosphodiesterase; (D) *Δ pdeH::kan, amyE::pdeH* showing active phosphodiesterase complementation and (E) *Δ ydaK dgcK dgcW dgcP::tet pdeH::kan* lacking a putative c-di-GMP phosphodiesterase and all possible diguanylate cyclases . Transitions *m/z* 691 → 152 (red), *m/z* 691 → 540 (blue) are not unique to c-di-GMP in *B. subtilis* lysates. Only two transitions are shown in insets to indicate relative abundance of the unique identifier (*m/z* 691 → 248 in black) and the most abundant fragmentation product (*m/z* 691 → 152 in red).

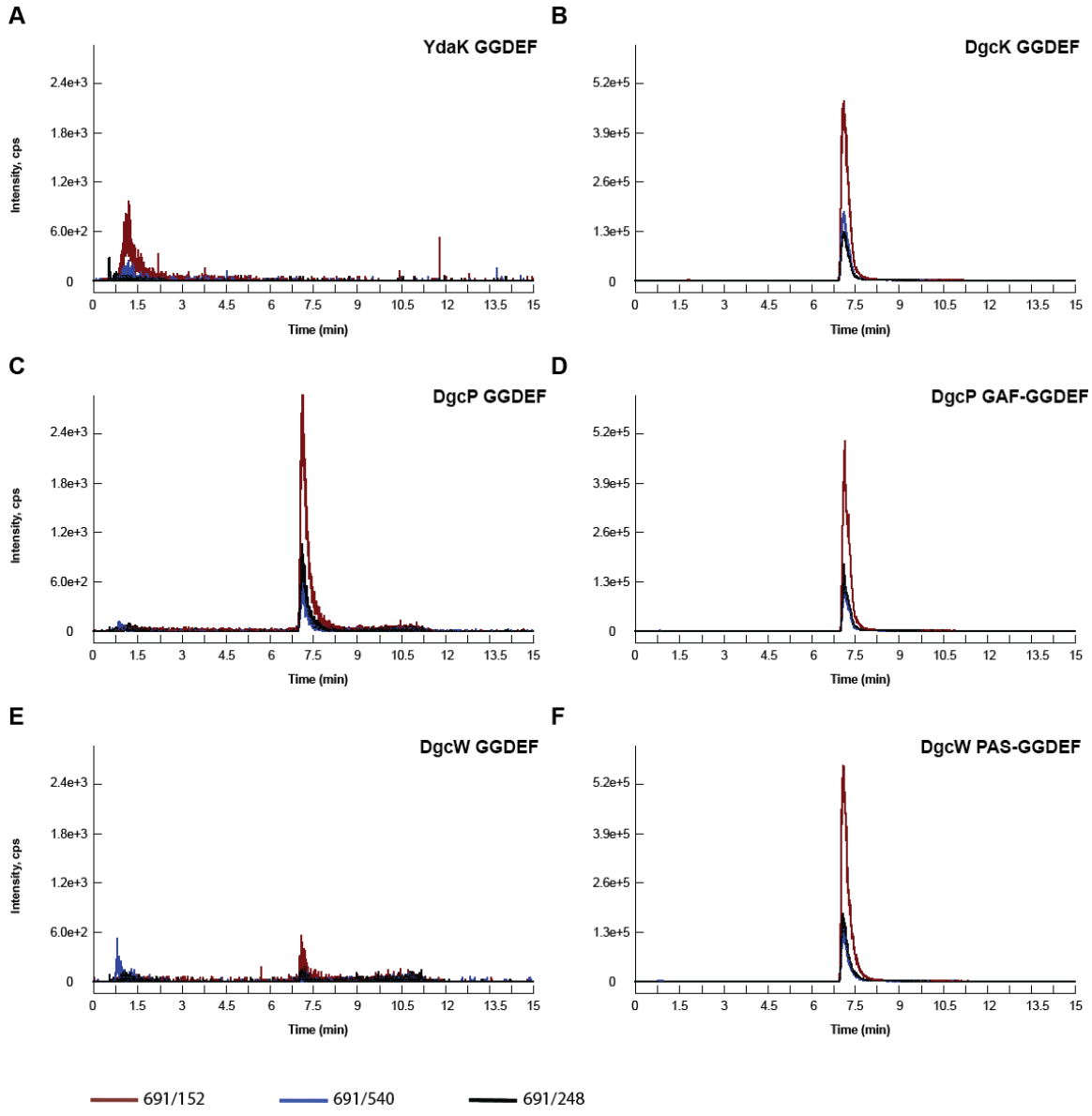


FIG. S4. *In vitro* detection of enzymatic activity of putative *B. subtilis* DGCs. To assess the activity of putative DGCs in vitro, LC-MS/MS was employed with MRM to detect enzymatic production of c-di-GMP from purified single or dual domain constructs containing GGDEF motifs. All ion peaks indicate production of c-di-GMP in *in vitro* assays. In total, four GGDEF single domain proteins were analyzed - (A) YdaK, (B) DgcK, (C) DgcP and (E) DgcW - as well as two dual domain constructs containing GGDEF and either GAF or PAS domains (D, F). As c-di-GMP co-purifies with the DgcK GGDEF protein exogenously expressed and purified from *E. coli*, uniformly labeled ^{13}C, ^{15}N-GTP was used as a substrate for the DgcK GGDEF enzymatic reactions. For this isotope, the MRM transitions *m/z* 721 →162 (red line), *m/z* 721→560(blue line) and *m/z* 721→ 263 (black line) were utilized (B), which

corresponds to the *m/z* 691 → 152 (red), *m/z* 691 → 540 (blue) and *m/z* 691 → 248 (black) transitions used in all other cases.

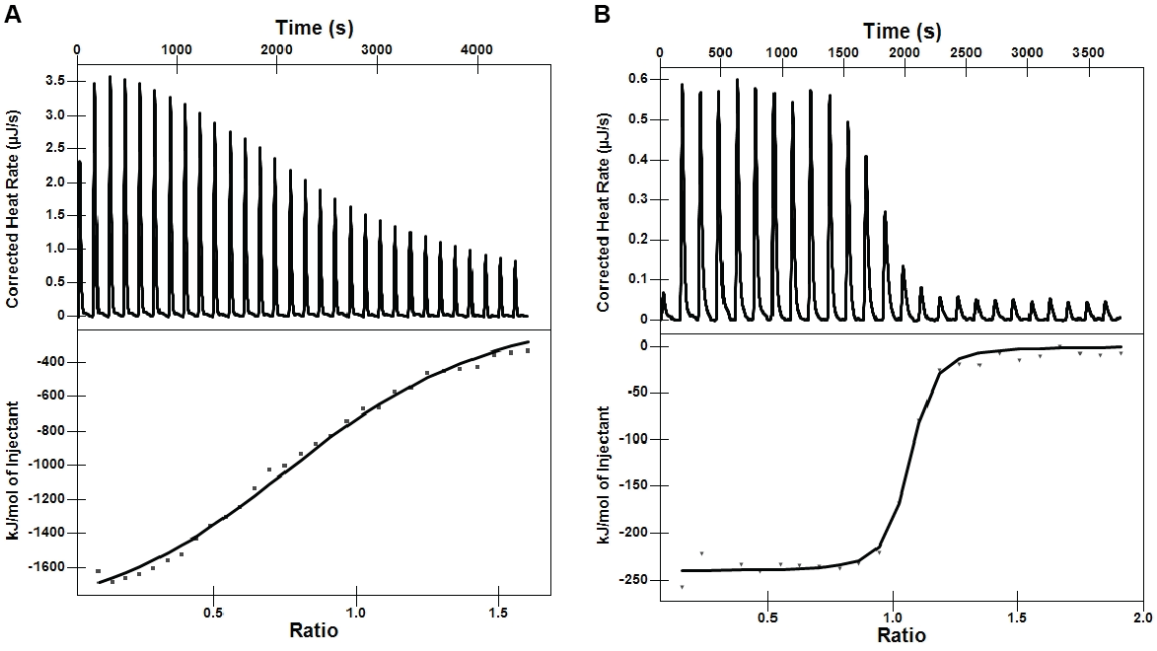


FIG. S5. Measurement of binding affinities of c-di-GMP to (A) YdaK and (B) DgrA. At least three titrations were carried out at 25 °C in each case with a single representative titration shown. The measured dissociation constant and standard deviations for c-di-GMP binding to YdaK and DgrA were 1.1 ± 0.2 µM and 11.0 ± 0.4 nM, respectively. Buffer conditions: 10 mM sodium citrate phosphate, 200 mM NaCl, pH 7.5.

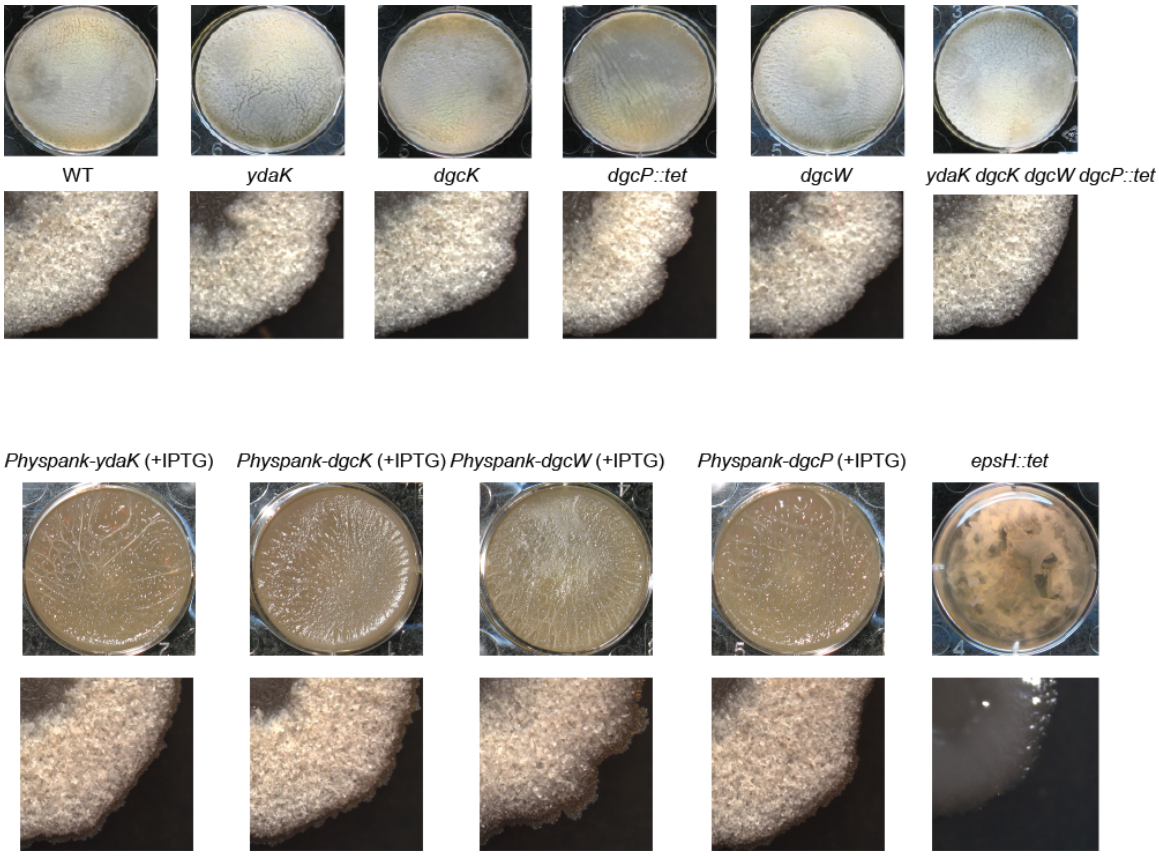


FIG. S6. *B. subtilis* biofilm formation is unaffected by alterations in c-di-GMP signaling. Strains with deletions or disruptions in any individual or all *B. subtilis* GGDEF protein(s) show no assembly defect in biofilm or pellicle formation assays, indicating c-di-GMP is not required for biofilm production (top two rows). Likewise, overexpression of each individual GGDEF protein does not alter biofilm or pellicle formation. The *epsH::tet* mutant is a control to illustrate a context in which these processes are disrupted.

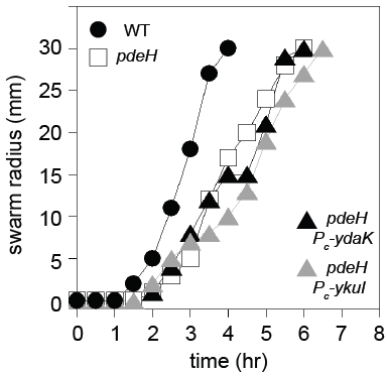
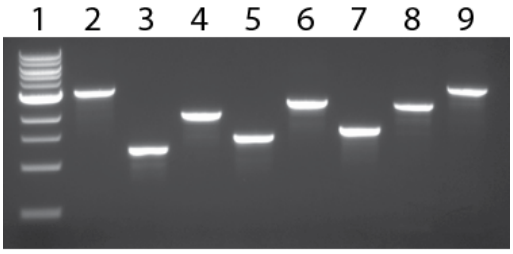


FIG. S7. Swarm expansion assays of strains constitutively expressing YdaK or YkuI, potential c-di-GMP receptors, in a background containing elevated c-di-GMP levels (*pdeH*). Swarming of *B. subtilis* 3610-wild type**;** DK391, a *pdeH* deletion; NPS265, a constitutive expression strain of *ykuI* in *pdeH* background; and NPS266, a constitutive expression strain of *ydaK* in *pdeH* were shown. YkuI and YdaK do not act as c-di-GMP receptors involved in motility, as each protein does not inhibit motility when expressed in a background containing c-di-GMP (*cf. dgrA* in Fig 9Q). Each point is an average of three replicates.



1. 1Kb DNA ladder
2. WT
3. Δ*dgcW* — Primer Pair 2928/2931
4. WT
5. Δ*ydaK* — Primer Pair 2932/2935
6. WT
7. Δ*dgcK* — Primer Pair 2936/2939
8. WT
9. *dgcP::tet* — Primer Pair 2924/2927

FIG. S8. PCR verification for dgc knockout mutants. Primer pairs for amplification of wild type and mutant sequences are listed on the right.

Table S1. *B. subtilis* strains used in this study

Strain	Genotype
3610	
DK391	*pdeH::kan*
DK392	*ΔydaK ΔdgcK ΔdgcW dgcP::tet pdeH::kan*
DK393	*pdeH::kan dgrA::erm*
DK404	*dgcP::tet pdeH::kan*
DK405	*ΔdgcW pdeH::kan*
DK406	*ΔydaK pdeH::kan*
DK407	*ΔdgcK pdeH::kan*
DS4856	*amyE::Physpank-dgcW spec*
DS4857	*amyE::Physpank-dgcP spec*
DS4858	*amyE::Physpank-ydaK spec*
DS4859	*amyE::Physpank-dgcK spec*
DS9883	*ΔdgcW*
DS9289	*ΔydaK*
DS9305	*ΔdgcK*
DS9537	*dgcP :: tet*
DS9408	*ΔydaK ΔdgcK*
DS9544	*ΔydaK dgcP::tet*
DS9545	*ΔdgcK dgcP::tet*
DS9548	*ΔydaK ΔdgcK dgcP::tet*
DS9884	*ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS079	*amyE::Physpank-dgcW spec ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS080	*amyE::Physpank-dgcP spec ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS081	*amyE::Physpank-ydaK spec ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS082	*amyE::Physpank-dgcK spec ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS207	*amyE::Physpank-dgcW ΔEAL spec , ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS208	*amyE::Physpank-dgcK GGGGF mutant spec, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS209	*amyE::Physpank-dgcP GGGGL mutant spec, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS215	*amyE::Physpank-dgcK GGAAF mutant spec, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS216	*amyE::Physpank-dgcP GGAAL mutant spec, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS217	*dgrA :: erm mls*
NPS218	*dgrA :: erm mls, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS219	*amyE::Physpank-dgcP spec , dgrA :: erm mls ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS220	*amyE::Physpank-dgcK spec, dgrA :: erm mls ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS221	*amyE::Physpank-dgcW ΔEAL spec dgrA :: erm mls ΔydaK ΔdgcK ΔdgcW dgcP::te*
NPS224	*amyE::Physpank-dgcW PAS-GGAAF ΔEAL spec ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS233	*amyE:: pdeH cat, pdeH::kan*
NPS235	*amyE::Pconstitutive-dgrA spec, pdeH::kan*
NPS236	*amyE::Pconstitutive-dgrA spec, pdeH::kan, ΔydaK ΔdgcK ΔdgcW dgcP::tet*

NPS247	*thrc::Physpank-dgcP mls, amyE::Pconstitutive-dgrA spec, pdeH::kan, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS252	*thrc::Physpank-$_{Bs\_dgcP}$ -dcpA mls ,amyE::Pconstitutive-dgrA spec, pdeH::kan, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS254	*thrc::Physpank-$_{Bs\_dgcP}$ -cd1420 mls ,amyE::Pconstitutive-dgrA spec, pdeH::kan, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS255	*thrc::Physpank-$_{Bs\_dgcP}$ -Atu1297 mls ,amyE::Pconstitutive-dgrA spec, pdeH::kan, ΔydaK ΔdgcK ΔdgcW dgcP::tet*
NPS265	*amyE::Pconstitutive-YkuI spec, pdeH::kan*
NPS266	*amyE::Pconstitutive-YdaK spec, pdeH::kan*

Table S2 Plasmids used in this study

Plasmid	Genotype/reference
pAH25	*amyE::spec amp* (gift from Dr. Amy Camp, Mount Holyoke College)
pAH52	*erm amp*
pDG1515	*tet amp* (Guérout-Fleury et al., 1995)
pDG364	*amyE cat*
pDP150	*thrc_ Physpank mls*
pDP391	*ΩΔdgcW mls amp*
pDP392	*ΩΔydaK mls amp*
pDP393	*ΩΔdgcK mls amp*
pDP407	*ypfA::erm amp*
pDR90	*amyE::Phyperspac spec amp* (gift from Dr. David Rudner, Harvard)
pDR111	*amyE intergration vector that contains Physpank, spec and amp*
pHis-parallel	N-terminal His-tag expression vector *amp*
pKB149	*amyE_Pconstitutive spec*
pMiniMAD	*ori^{BsTs} mls* (Patrick and Kearns, 2008)
pMBP-*parallel*	N-terminal MBP-tag expression vector *amp*
pHS001	*pHis-parallel with pdeH, amp*
pXG075	*pHis-parallel with ydaK GGDEF domain, amp*
pXG076	*pHis-parallel with dgcK GGDEF domain, amp*
pXG061	*pMBP-parallel with dgcP GGDEF domain, amp*
pXG074	*pHis-parallel with dgcP GAF-GGDEF domain, amp*
pXG077	*pMBP-parallel with dgcW GGDEF domain, amp*
pXG079	*pHis-parallel with dgcW PAS-GGDEF domain, amp*
pXG001	*amyE::Physpank-dgcW, spec*
pXG002	*amyE::Physpank-dgcP, spec*
pXG003	*amyE::Physpank-ydaK, spec*
pXG004	*amyE::Physpank-dgcK, spec*
pXG085	*pHis-parallel with dgrA, amp*
pXG086	*amyE::Physpank-dgcW ΔEAL, spec*
pXG087	*amyE::Physpank-dgcK GGGGF mutant, spec*
pXG088	*amyE::Physpank-dgcP GGGGL, spec*
pXG090	*amyE::Physpank-dgcK GGAAF, spec*
pXG091	*amyE::Physpank-dgcK GGAAF, spec*
pXG092	*amyE::Physpank-dgcW GGAAF ΔEAL, spec*
pXG093	*pHis-parallel with dgrA C4, amp*
pXG094	*amyE:: pdeH, cat (complementation)*
pXG095	*amyE::Pconstitutive-dgrA, spec*
pXG100	*thrC::Physpank-dgcP, mls*
pXG101	*thrC::Physpank-$_{dgcP}$, mls*
pXG102	*thrC::Physpank-$_{dgcP}$-dcpA, mls*
pXG104	*thrC::Physpank-$_{dgcP}$-CD1420, mls*
pXG105	*thrC::Physpank-$_{dgcP}$-Atu1297, mls*

pXG124	*amyE::Pconstitutive-ykuI, spec*
pXG125	*amyE::Pconstitutive-ydaK, spec*

Table S3 Primers used in this study

Primers for enzymatic activity assays *in vitro*		
GXH466	YdaK GGDEF domain(NcoI)	ggg ccatgg atcacgatattacagcagag
GXH460	YdaK GGDEF domain(EcoRI)	ggg gaattc ttatagttcattcatcatcatctcgttttcc
GXH467	DgcK GGDEF domain (NcoI)	ggg ccatgg atcatgaaacaaatgcac
GXH462	DgcK GGDEF domain(EcoRI)	ggg gaattc tcattctttttttctgaaaaacacactctg
GXH454	DgcP GGDEF (NcoI)	ggg ccatgg tgaaaacagatcagctgactg
GXH440	DgcP GGDEF(EcoRI)	ggg gaattc tcatgagtcatgaatcatcaagcg
GXH465	DgcP GAF-GGDEF (NcoI)	ggg ccatgg atcaggaaagactctataac
GXH458	DgcP GAF-GGDEF(EcoRI)	ggg gaattc ttattttattgagtcatgaatcatcaagcgg
GXH453	DgcW GGDEF (NcoI)	ggg ccatgg ctcattatgattcgcttacagac
GXH471	DgcW GGDEF (EcoRI)	ggc gaattc ttaagaatagtatctgtatttgcttttg
GXH464	DgcW PAS-GGDEF (stuI)	gggaa aggcct aaatattgatgcaatttttattt
GXH468	DgcW PAS-GGDEF (SpeI)	ggc actagt ttaagaatagtatctgtatttgcttttg
GXH480	PdeH (NcoI)	gggg ccatgg gaagggtgtttgttgcaagacag
GXH481	PdeH (EcoRI)	gggg gaattc tcattttgcgtccataagattatgacac
GXH482	DgrA(NcoI)	ggg ccatgg gaatagagattggagaaaatgtacttttag
GXH483	DgrA (EcoRI)	gggg gaattc ttattccattcgggcctttcttc
GXH513	DgrA (EcoRI)	gggg gaattc ttactttcttctcttatttaactggcgc
Primers for overexpression/ constitutive expression *in vivo*		
GXH301	dgcWF (SalI)	aggaggtcgacgtcaacaagactggaacacacgat
GXH302	dgcWR (NheI)	ctcctgctagcaaggagtacgattcatgtgtagtaa
GXH303	dgcPF(NheI)	aggaggctagcgcttcaccgtttatgatataataaac
GXH304	dgcPR(SphI)	ctcctgcatgcgacgaaattgatgaaaaatacttaact
GXH305	ydaKF(NheI)	aggaggctagcacgcattcatttgacaatctgctg
GXH306	ydaKR(SphI)	ctcctgcatgcaatagaagcatcatgacgcatagc
GXH307	dgcKF(HindIII)	aggagaagcttttccgatgggtcaagtgcgaatt
GXH308	dgcKR(NheI)	ctcctgctagcaagaatatcagggagatcttgtcc
GXH516	dgrAF (SphI)	gggg gcatgcgtgagtatattgaatgcgaaagagtgaag
GXH517	dgrAR (BamHI)	gggg ggatccggaagtgtttgccgatgtttattcc
GXH532	dgcP (HindIII)	attgtgagcggataacaattaagcttgcttcaccgtttatgatata ataaac
GXH533	dgcP (SphI)	cgtttccaccgaattagcttgcatgcgacgaaattgatgaaaaat acttaact
GXH534	dgcP(-60~+3)	attgtgagcggataacaattaagc
GXH535	dgcP (-60~+3)	tttattatatcataaacggtgaagcaagcttaattgttatccgctca ca
GXH536	dgcP (-60~+3)	ttgcttcaccgtttatgatataataaacaaaaattagtggtaaactt ttaggtg
GXH537	dgcP (-60~+3)	cactagtgctagccatgtttttatcacctaaaagtttaccactaattt ttg
GXH538	dgcP (-60~+3)	ataaaaacatggctagcactagtgcatgcaagctaattcggtgga

GXH539	dgcP (-60~+3)	gacctcgtttccaccgaattagcttgcat
GXH540	dcpA (NheI)	tggtaaacttttaggtgataaaaacatggctagcattagatattca actcgtcccatcct
GXH521	dcpA (SphI)	cgtttccaccgaattagcttgcatgctggtcgtgaggaagcccaaa aaggtgaag
GXH542	CD1420 (NheI)	tggtaaacttttaggtgataaaaacatggctagctttaaagaaatt ttttaagaacttttccagga
GXH543	CD1420 (SphI)	gacctcgtttccaccgaattagcttgcatgcatgtacaagtttaaaa aaagaaagataattttaatt
GXH544	Atu1297 (NheI)	tggtaaacttttaggtgataaaaacatggctagcacggcgagagt tctggttgttgac
GXH531	Atu1297 (SphI)	cgtttccaccgaattagcttgcatgcgagaggctgcgcaaacagtc gtggttaa
GXH583	ykuI (SphI)	ggg gcatgc gtgcgtataatcagacaaagatagagaagc
GXH584	ykuI (NheI)	ggg gctagc aaacggcctttcagccgttaaatc
GXH585	ydaK(SphI)	ggg gcatgc cacgcattcatttgacaatctgctg
GXH586	ydaK(NheI)	ggg gctagc aatagaagcatcatgacgcatagc

Primers for gene knockouts and complementation *in vivo*

2006		cgccattcgccagggctgcag
2007		ctcctgcatgcacacacattatgccacaccttgtag
2928	dgcWUF (EcoRI)	aggaggaattcatcctgccaaaacaacgcca
2929	dgcWUR (XhoI)	ctcctctcgagatattccatcggcatttccat
2930	dgcWDF (XhoI)	aggagctcgaggtgattgccgaaggtgtgg
2931	dgcWDR (BamHI)	ctcctggatcccagggtataggccttcgt
2932	ydaKUF (EcoRI)	aggaggaattcgtctttgaagacatacaatatg
2933	ydaKUR (XhoI)	ctcctctcgagcagagcaccttctgattgct
2934	ydaKDF (XhoI)	aggagctcgagggaaagtatgtgtcgcttacg
2935	ydaKDR (BamHI)	ctcctggatccctctgagctgtttcgccac
2936	dgcKUF(EcoRI)	aggaggaattctcgttgaagtcggtctagctc
2937	dgcKUR (XhoI)	ctcctctcgagtactagcctttctttaaacagg
2938	*dgcK* DF (XhoI)	aggagctcgagcttgccgatcaaatgctttac
2939	*dgcK* DR (BamHI)	ctcctggatcctcgggattgctgagtctgac
3037	*dgcP* UP F 3 (BamHI)	aggagggatccgtctgctctattcgaccatg
3038	*dgcP* UP R 3 (PstI)	ctcctctgcaggcacgcaagatggaaacgc
3039	*dgcP* DN F 3 (EcoRI)	aggaggaattcacgtacggccatcagactg
3040	*dgcP* DN R 3 (XhoI)	ctcctctcgagcagaaattgtgcttccgattc
GXH489	*dgrA* UPF (EagI)	aggagcggccggaacaggaagcaaaacaaattg
GXH490	*dgrA* UPR (BamHI)	ctcctggatccgtacattttctccaatctctatc
GXH491	*dgrA* DNF (XhoI)	aggagctcgagcaggctttgcttcaatactgc
GXH492	*dgrA* DNR (KpnI)	ctcctggtacctctgcatttggcagcacatg
3428	*pdeH_kan* ITA UP F	ggaaagcgctttaatatcccg

3429	*pdeH_kan* ITA UP R	caattcgccctatagtgagtcgtacaaacaccctcattgatttatc
3430	*pdeH_kan* ITA DN F	ccagcttttgttccctttagtgagatggacgcaaaatgattgcgg
3431	*pdeH_kan* ITA DN R	ggacatttaaaggacagcagg
GXH514	*pdeH* complementation F	tgataagctgtcaaacatgagaattcgaaagaaagtctaccgca ttattaatcagtcc
GXH515	*pdeH* complementation R	atggtagcgaccggcgctcaggatcctttgcggtggtcagagga ccgc

Primers for DGC mutants *in vivo*

GXH484	dgcW in vivo truncation	cctc gctagc ttaagaatagtatctgtatttgcttttg
GXH485	dgcK GGGGF mutant	gatcctgccgctcggatcggcggtggcggttttgccgtgctcctg ccgaactg
GXH486	dgcK GGGGF mutant	cagttcggcaggagcacggcaaaaccgccaccgccgatccga gcggcaggatc
GXH487	dgcP GGGGL mutant	cggtgcccgctggggaggaggaggactggcgatttatttgccaa atgtgccg
GXH488	dgcP GGGGL mutant	cggcacatttggcaaataaatcgccagtcctcctcctccccagcg ggcaccg
GXH495	dgcK GGAAF mutant	gatcctgccgctcggatcggcggtgccgcttttgccgtgctcctg ccgaactg
GXH496	dgcK GGAAF mutant	cagttcggcaggagcacggcaaaagcggcaccgccgatccga gcggcaggatc
GXH497	dgcP GGAAL mutant	cggtgcccgctggggaggagccgccctggcgatttatttgccaa atgtgccg
GXH498	dgcP GGAAL mutant	cggcacatttggcaaataaatcgccagggcggctcctccccagc gggcaccg
GXH499	dgcW GGAAF ΔEAL	ccgtcttggcggtgctgcatttattattatattaac
GXH500	dgcW GGAAF ΔEAL	gttaatataataataaatgcagcaccgccaagacgg