Supplemental Material

Single cell microscopy reveals that levels of cyclic di-GMP vary among *Bacillus subtilis* subpopulations

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Supplemental Material Contents:

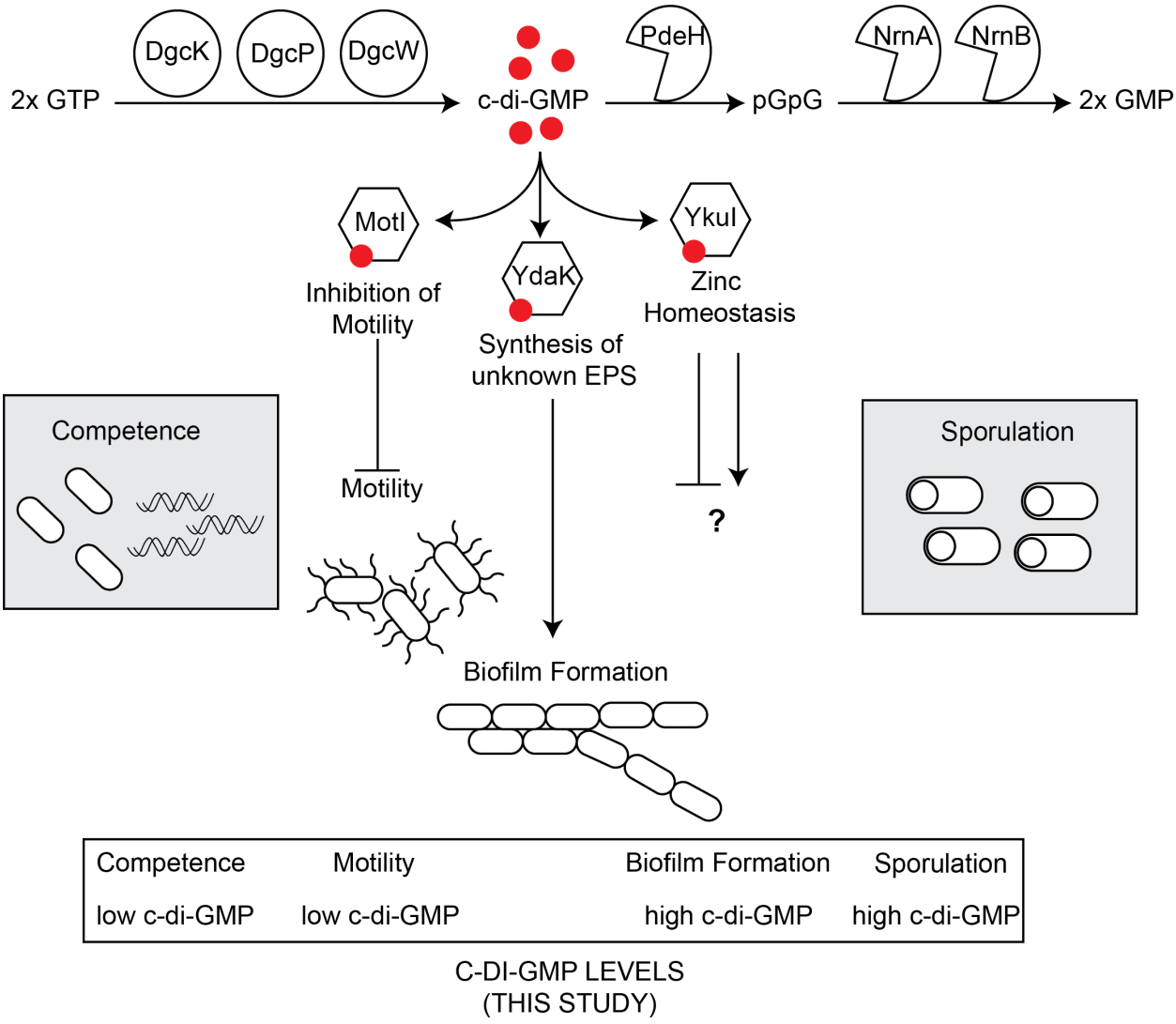

Figure S1. Schematic diagram of c-di-GMP regulation in *B. subtilis.* Diguanylate cyclases (DgcK, DgcP, DgcW) synthesize c-di-GMP from 2 GTP molecules. Phosphodiesterases (PdeH) hydrolyze c-di-GMP to pGpG, which is in turn hydrolyzed to 2 GMP molecules by RNases NrnA and NrnB. C-di-GMP binds effectors (MotI, YdaK, YkuI), which target cellular outcomes.

A. DNA Sequence of P_{T7}-*lchAA* aptamer-Spinach2 used for fluorescence activation assays

5'-TAATACGACTCACTATAGGGCCCGGATAGCTCAGTCGGTAGAGCAGCGGCCG**G**
ATGTAACTGAATGAAATGGTGAAGGACGGGTCCAAT*GATAAAGGCAAACCTGCGGAAACGCAGGG*
*ACGCAAAGCCATGGCCTAAGGTGCTGACGGTGCTACGGTTGACAGGTTGCC*AT**TTGTTGAGTAGAG**
TGTGAGCTCCGTAACTAGTTACATCCGGCCGCGGGTCCAGGGTTCAAGTCCCTGTTCGGGCGCCA

B. DNA template sequence of P_{const}-*lchAA* leader used for *in vitro* transcription termination assays

5'-CTACATCCAGAACAACCTCTGCTAAAATTCCTGAAAAATTTTCGAAAAAGTTGTT
GACTTTATCTACAAGGTGTGGCATAATGTGTGTGCA*GCAGAAAATGAATTTATATCAAGAAAAGCAGA*
TAAAGGCAAACCTGCGGAAACGCAGGGACGCAAAGCCATGGCCTAAGGTGCTGACGGTGCTACGGT
TGACAGGTTGCCGAATAAACAGGGAGTTCGCCCGTTTTTATTCGGGCGGGCTCTTTTCTTTTTATTTC
CAATATAATGTTTTATTGGAAACGACAAATCTGTGACAGCGTTTTTCG

C. DNA sequence of P_{const}-*lchAA* leader-*yfp* reporter used for fluorescence microscopy

5'-GTAGCCCTTGCCTACCTAGCTTCCAAGAAAGATATCCTTACAGCACAAGAGCGG
AAAGATGTTTTGTTCTACATCCAGAACAACCTCTGCTAAAATTCCTGAAAAATTTTCGAAAAAGTTGTT
GACTTTATCTACAAGGTGTGGCATAATGTGTGTGCA*GCAGAAAATGAATTTATATCAAGAAAAGCAGA*
TAAAGGCAAACCTGCGGAAACGCAGGGACGCAAAGCCATGGCCTAAGGTGCTGACGGTGCTACGGT
TGACAGGTTGCCGAATAAACAGGGAGTTCGCCCGTTTTTATTCGGGCGGGCTCTTTTCTTTTTATTTC
CAATATAATGTTTTATTGGAAACGACAAATCTGTGACAGCGTTTTTCGCTCATCGCAAAACCGCAACAT
*TGCATTGCGGCTTGGCTGTTCGCATCGTCATACATAACAAGAGAT*AAGCTTAAGGAGGAAAGTCACA
TT**ATGAGCAAAGGTGAAGAACTGTTCACCGGCGTTGTGCCAATTCTGGTTGAGCTGGATGGTGACG**
TGAATGGCCACAAATTTTCCGTGTCTGGTGAAGGCGAGGGTGATGCTACTTATGGCAAACTGACTC
TGAAACTGATCTGTACCACCGGCAAACTGCCTGTTCCGTGGCCAACTCTGGTCACTACTCTGGGTT
ACGGCCTGATGTGTTTTGCGCGTTACCCGGATCACATGAAACAGCATGACTTCTTCAAATCTGCCA
TGCCGGAAGGCTATGTCCAAGAACGTACGATCTTTTTCAAGGACGACGGCAACTATAAAACCCGT
GCCGAAGTTAAATTCGAGGGTGACACCCTGGTCAACCGCATCGAACTGAAAGGCATTGACTTCAA
AGAGGACGGCAACATTCTGGGTCACAAGCTGGAATACAACTACAACTCCCACAACGTTTACATTAC
TGCTGACAAGCAGAAAAACGGCATCAAAGCAAACTTCAAGATCCGTCACAACATTGAAGATGGTG
GCGTACAGCTGGCAGATCACTACCAGCAGAACACTCCAATCGGTGATGGCCCAGTACTGCTGCCA
GATAACCATTACCTGTCCTACCAGAGCAAACTGTCTAAAGACCCGAACGAAAAACGTGACCACAT
GGTACTGCTGGAATTTGTTACCGCGGCAGGCATTACCCACGGTATGGACGAACTGTATAAATAAGC
TAGCAAAAACCCCGCCCCTGACAGGGCGGGGTTTTTTTT-3'

Figure S2. DNA sequences of (A) the *lchAA* riboswitch aptamer-Spinach2 construct, transcribed from a T7 promoter, (B) the *lchAA* riboswitch "run-off" construct used for *in vitro* transcription under expression of a bacterial P_{const} promoter, and (C) the constitutively expressed *lchAA* riboswitch-*yfp* reporter construct used for all *in vivo* studies. For each sequence, the promoter is underlined at the 5' end, and all *lchAA* leader sequences are italicized. In panel A, Spinach2 is in bold. Additionally, a $tRNA^{Lys}$ scaffold sequence was added to aid in mRNA stability. In panel B, the terminator from the *lchAA* leader is also underlined. In panel C, the *yfp* ORF is in bold.

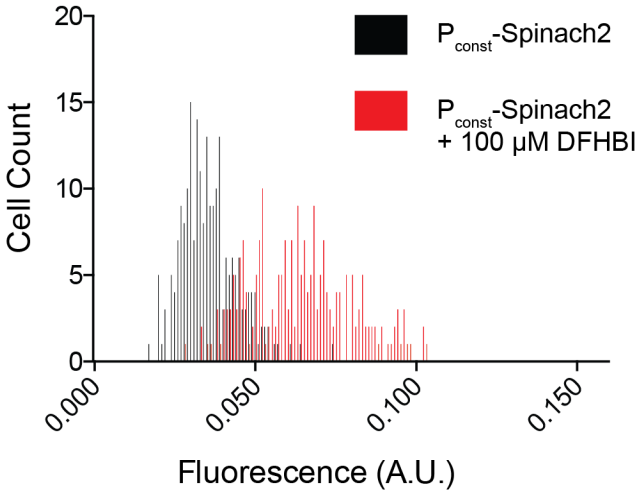

Figure S3. Quantification of the fluorescence intensity per cell of P_{const}-Spinach2 with the addition of 100 µM DFHBI, compared to untreated cells, expressed in *B. subtilis* PY79 wild-type (n~300).

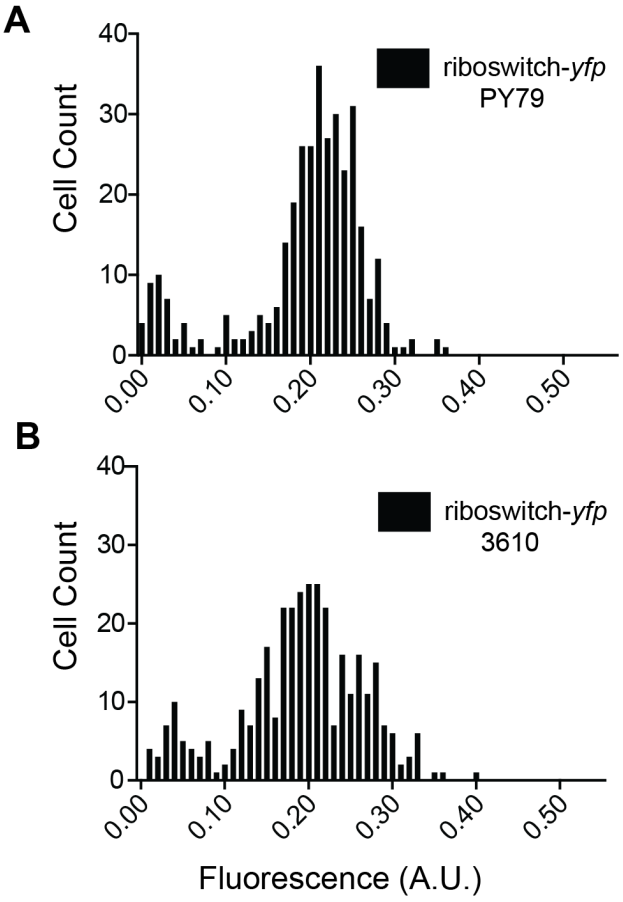

Figure S4. Quantification of fluorescence intensity per cell of P_{const}-*lchAA* UTR-*yfp* in *B. subtilis* wild-type strains (A) PY79, or (B) 3610, (n~350).

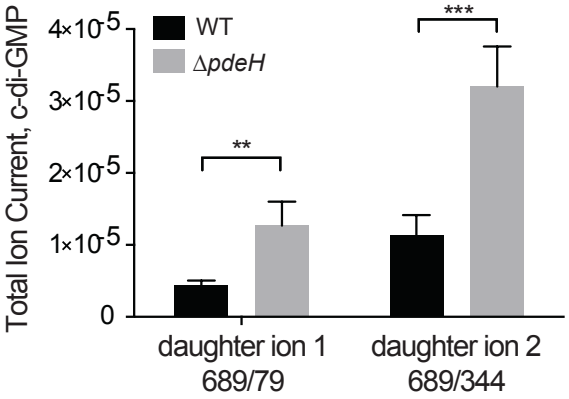

Figure S5. Quantification by LC-MS/MS of intracellular c-di-GMP levels in *B. subtilis* PY79 wild-type (WT) or Δ*pdeH* in late-log phase (OD_{600}=1.0). Results were obtained for two daughter ions. Error bars represent mean ± SD (n=3). Statistical significance determined by unpaired two-tailed Student's *t*-test.

Table S1.

Strain	Genotype	Antibiotic Marker	Source
PY79[a]	Prototrophic derivative of wild-type *B. subtilis* 168		(1)
DS7187	3610 Δ*comI*		(2)
Weiss9	*amyE* [b]::P_{const}-*yfp*	*cat*	This study
Weiss65	3610 Δ*comI amyE* ::P_{const}-*lchAA* leader-*yfp*	*cat*	This study
Weiss70	*amyE* ::P_{const}-*lchAA* leader-*yfp*	*cat*	This study
Weiss96	*amyE* ::P_{hagA} -*gfp*	*cat*	This study
	thrC [c] *::P* $_{const}$-*lchAA leader-mCherry*	*mls*	
Weiss97	*amyE* ::P_{tapA} -*yfp*	*cat*	This study
	thrC::P $_{const}$-*lchAA leader-mCherry*	*mls*	
Weiss117	*amyE* ::P_{const}-*lchAA* leader$^{\Delta apt(1\text{-}65\ nt)}$-*yfp*	*cat*	This study
Weiss162	*amyE* ::P_{const}-*lchAA* leader-*yfp*	*cat*	This study
	Δ*pdeH::mls*	*mls*	
Weiss163	*amyE* ::P_{const}-*yfp*	*cat*	This study
	Δ*pdeH::mls*	*mls*	
Weiss171	Δ*pdeH::mls*	*mls*	This study
Weiss173	*amyE* ::P_{comG} -*yfp*	*cat*	This study
	thrC ::P_{const}-*lchAA* leader-*mCherry*	*mls*	
Weiss209	Δ*pdeH amyE* ::P_{tapA} -*yfp*	*cat*	This study
	thrC ::P_{const}-*lchAA* leader-*mCherry*	*mls*	
Weiss210	Δ*pdeH amyE* ::P_{comG} -*yfp*	*cat*	This study
	thrC ::P_{const}-*lchAA* leader-*mCherry*	*mls*	
Weiss211	Δ*pdeH amyE* ::P_{hagA} -*gfp*	*cat*	This study
	thrC ::P_{const}-*lchAA* leader-*mCherry*	*mls*	
Weiss246	*amyE* ::P_{const}-spinach2-$tRNA^{Lys}$scaffold	*cat*	This study
Weiss273	Δ*sinR amyE* ::P_{hagA} -*gfp*	*cat*	This study
	thrC::P $_{const}$-*lchAA leader-mCherry*	*mls*	
Weiss274	Δ*spo0A amyE* ::P_{hagA} -*gfp*	*cat*	This study
	thrC::P $_{const}$-*lchAA leader-mCherry*	*mls*	
Weiss275	Δ*sigD amyE* ::P_{hagA} -*gfp*	*cat*	This study
	thrC::P $_{const}$-*lchAA leader-mCherry*	*mls*	
Weiss287	*amyE*::P_{pdeH}-*sf gfp*	*cat*	This study
Weiss288	Δ*spo0A amyE*::P_{pdeH}-*sf gfp*	*cat*	This study
Weiss290	Δ*sinR amyE*::P_{pdeH}-*sf gfp*	*cat*	This study
Weiss292	Δ*sigD amyE*::P_{pdeH}-*sf gfp*	*cat*	This study

[a] All strains are derivatives of PY79 unless otherwise noted
[b] Integration at *amyE* locus was performed with plasmids derived from pDG1662 (Bacillus Genetic Stock Center)
[c] Integration at *thrC* locus was performed with plasmids derived from pDG1664 (Bacillus Genetic Stock Center)

Supplemental Materials and Methods

Fluorescence microscopy & quantification

Single colonies were used to inoculate liquid MSgg medium (3) and grown at 37˚C shaking overnight. The following morning, cultures of each strain were inoculated 1:50 in fresh medium and grown at 37˚C shaking until reaching an optical density at 600 nm (OD_{600}) of 1.0. To assess Spinach2 fluorescence, DFHBI was added to each culture to achieve a final concentration of 100 μM and cultures were allowed to shake at 37˚C in the dark for an additional hour. Otherwise, aliquots of these cultures were then placed on 1.5% low-melting agarose MSgg pads and allowed to dry for 10 minutes. Agarose pads were inverted onto a glass bottom dish (Willco Wells). Cells were imaged at room temperature using a Zeiss Axio-Observer Z1 inverted fluorescence microscope, equipped with a Rolera EM-C_2 electron-multiplying charge-coupled (EMCC) camera, enclosed within a temperature-controlled environmental chamber. Fluorescence intensity per cell was quantified using Oufti analysis software (4). Images were analyzed and adjusted with FIJI software (5).

Metabolite extraction and quantification of c-di-GMP in B. subtilis

Three independent replicates of *B. subtilis* PY79 WT and Δ*pdeH::mls* were grown overnight in liquid MSgg medium (3) shaking at 37˚C. The following day cultures of each strain were inoculated (1:50) into fresh MSgg and agitated at 37˚C until reaching an optical density at 600 nm (OD_{600}) of 1.0. Metabolite extraction was performed as described previously (6). 5 mLs of each culture were passed through 0.2 μm nylon filters (EMD Millipore). Metabolites were extracted by inverting the filters into petri dishes that contained 1.5 mL pre-chilled extraction solvent composed of 40:40:20 acetonitrile/methanol/water. Dishes were placed on dry ice for 15 minutes before the wash was collected and spun at max speed for 5 minutes at 4˚C. The supernatant was then placed in a vacuum centrifuge until metabolite extracts were dry. Detection of c-di-GMP by LC-MS/MS was previously described (7). Briefly, bacterial extract was resuspended in Solvent A (10 mM tributylamine in water, pH 5.0) and centrifuged twice to remove insoluble particles. Metabolites were then separated on a

Synergi Fusion-RP column (4 µm particle size, 80 Å pore size, 150 mm x 2 mm, Phenomenex) using a Shimadzu high performance liquid chromatography machine and simultaneously analyzed by a triple quadrupole mass spectrometer (3200 QTRAP, ABSCIEX). The total run time was 20 min at a binary flow rate of 0.5 ml min^{-1}, with 10 mM tributylamine in water (pH 5.0) as Solvent A and 100% methanol as Solvent B. The following gradient was performed: 0.01 min, 0% B, 4 min, 0% B, 11 min, 50% B, 13 min, 100% B, 15 min, 100% B, 16 min, 0% B, 20 min, 0% B. C-di-GMP and pGpG were detected by multiple reaction monitoring (MRM) under negative mode using the ion pairs 689/79 and 689/344 (c-di-GMP). C-di-GMP was quantified using the Analyst® software (version 1.6.2) by calculating the total peak area and normalized by total ion current (TIC). Authentic c-di-GMP standards were injected and analyzed alongside samples.

Supplemental References

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