SUPPLEMENTAL MATERIAL

A nutrient-regulated cyclic diguanylate phosphodiesterase controls *Clostridium difficile* biofilm and toxin production during stationary phase

Erin B. Purcell[1], Robert W. McKee[1], David S. Courson[2], Elizabeth M. Garrett[1], Shonna M. McBride[3], Richard E. Cheney[2], and Rita Tamayo[1*]

[1]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC, USA

[2]Department of Cell Biology and Physiology, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC, USA

[3]Department of Microbiology and Immunology, Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA, USA

Table S1. Strains and plasmids used in this study.

Name	Description	Reference
E. coli		
DH5α	F- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR*17(rκ -, mκ+) *phoA supE*44 *thi*-1 *gyrA*96 *relA1* λ- *tonA*	Invitrogen, (1)
RT881	DH5α pMC234	This study
RT899	DH5α pMC234::*pdcA::catP*	This study
RT1099	DH5α pRT1099	This study
RT1214	DH5α pRT1214	This study
BL21	*fhuA2 [lon] ompT gal [dcm] ΔhsdS*	NEB
RT489	BL21 pMMBneo::*pdcA*	(2)
RT548	BL21 pMMBneo::*pdcA*(GA)	This study
RT549	BL21 pMMBneo::*pdcA*-ΔPAS	This study
RT496	BL21 pMMBneo::*pdcA*-EAL	(2)
HB101	F^- *mcrB mrr hsdS20*(r_B^- m_B^-)*recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*	(3)
HB101(pRK24)	*E. coli* strain used in conjugations with *C. difficile*	(3)
RT679	HB101(pRK24) pPdcA(GA)	This study
RT680	HB101(pRK24) pPdcA-ΔPAS	This study
RT925	HB101(pRK24) pMC234::*pdcA::catP*	This study
RT1177	HB101(pRK24) pRT1099	This study
RT1232	HB101(pRK24) pRT1214	This study
C. difficile		
JIR8094	erythromycin-sensitive derivative of 630	(4)
	JIR8094::pSD21 (*codY*-null)	(5)
630	Wild-type 630, ribotype 012	(6)
RT476	630 pMC-Pcpr	(2)
RT478	630 pMC-Pcpr::*pdcA*	This study
RT959	630 *pdcA*::*catP*	This study
RT1234	630 pRT1099	This study
RT1235	630 *pdcA::catP* pRT1099	This study
RT1237	630 *pdcA::catP* pRT1214	This study
RT1668	630 *pdcA::catP* pRT1662	This study
Plasmids		
pMMBneo	Low copy expression vector, Ptac promoter, *neo* cassette	(7)
pMMBneo::*pdcA*	*pdcA*-His6	(2)
pMMBneo::*pdcA*-EAL	*pdcA*(Δ1-438)-His6 (EAL domain only)	(2)
pMMBneo::*pdcA*(GA)	*pdcA(GA)*-His6 (full length PdcA with residues 350-354 (DGDEM) mutated to AAAAA)	This study
pMMBneo::*pdcA*ΔPAS	*pdcA*(Δ1-251)-His6 (GGDEF and EAL domains remain)	This study
pMC123	*E. coli- C. difficile* shuttle vector, *bla, catP*	(3)
pMC-Pcpr	pMC123 with nisin-inducible *cprABCK* promoter	(2)
pPdcA	pMC-Pcpr::*pdcA* (CD630_15150)	(2)
pPdcA-EAL	pMC-Pcpr*::pdcA*-EAL (EAL domain only)	(2)

pPdcA(GA)	pMC-Pcpr::*pdcA*(GA) (DGDEM to AAAAA mutations)	This study
pPdcAΔPAS	pMC-Pcpr:: *pdcA*ΔPAS (GGDEF and EAL domains remain)	This study
pMC95	pUC19 with oriT	(8, 9)
pMC234	*C. difficile* allelic exchange plasmid, pMC95 with *catP* in *Bam*HI site of MCS	This study
pMC234::*pdcA:: catP*	*pdcA* upstream and downstream flanking sequences flanking the *catP* gene in pMC234	This study
pBTS	*aad9* locus cloned into an *E. coli/S. aureus* shuttle vector	(10)
pRT1099	pMC123 with *catP* replaced with *aad9*	This study
pRT1214	pRT1099 with P_{pdcA}-*pdcA*	This study
pRT1662	pRT1099 with P_{pdcA}-*pdcA*(E479A)	This study
pEAV1	*C. difficile codY* with 6× histidine-tag cloned in pBAD30, Ap	(5)

Table S2- Oligonucleotides used in this study.

Name	**Sequence (5' to 3')***	**Reference**
pdcAF	CAGGTACCTTTAGGATACATTTTTATGAACAAACATAATTTTGAAGTTATATTAAATC	(2)
pdcAR	CCCTGCAGCTAATGGTGATGGTGATGGTGATTATCTAGCTTTAAAAGGTCAAAGATTTC	(2)
pdcAgaR	CTTTATAAACTT**GCAGCAGCAGCAGCA**GGCATTCTTGTTGATAATG	This study
pdcAgaF	CAACAAGAATGCC**TGCTGCTGCTGCTGC**AAGTTTATAAAGTTCAGC	This study
pdcAeaF	ATAGGTGTAG**C**AGTTCTTTTAAGG	This study
pdcAeaR	CCTTAAAAGAACT**G**CTACACCTAT	This study
pdcAdpF	CTAGGTACCTTTAGGATACATTTTTATGCGTGATGAATTTGGAGAAC	This study
pdcAFbamHI	CAGGATCCGAGCAGTAACTATGGAGGAG	This study
pdcARpstI	GACCTGCAGATACTATTCCCAATTTAACATCC	(2)
67EHF	CGACATCATAACGGTTCTGG	(11)
67EHR	TTCACTTCTGAGTTCGGCAT	(11)
pUCmcsF	TCTTCGCTATTACGCCAG	(8)
m13R	AACAGCTATGACCATG	(12)
tcdBqF	AAGGAATATCTAGTTACAGAAGTATTAGAGC	(13)
tcdBR	GCAGTGTCATTTATTTGACCTCCA	(13)
oMC15	GCAGGCCCTCGGATCTTTTCCGCTGCA	This study
oMC16	GCGACGTCCTTATCGGCCAGCCTCG	This study
oMC2	GCGGATCCTAGCGCCTACGGGGAATT	This study
oMC143	GCGGATCCCCTTGGTTGTGTTGCTTTTCG	This study
pdcAf1	CAAGAATTCACCTTAAACCCACCAAATG	This study
pdcAr1	CAAGGTACCCATATACTTATCTCCTCCATAGTTAC	This study
pdcAf2	CAAGTCGACGATAATTAATTCTAATTTATCAATAATATCACTG	This study
pdcAr2	CAACTGCAGCCTCTAAACTCATTCCTATTTCTC	This study

pdcAr0	AGCTGGGGAGTTCTATTTTG	This study
blaF	CATGAGATTATCAAAAAGGATCTTC	This study
blaR	GCCTTCCTGTTTTTGCTCAC	This study
aad9F_EcoRV	GATATCGTAACGTGACTGGCAAGAG	This study
aad9R_EcoRV	GATATCACCCAAAATTGAAAAAAGTGTTTCC	This study
pdcApromF	AAAGCATGCACCATCCATAAATATCTTACCA	This study
pdcApromR	GACGGATCCATACTATTCCCAATTTAACATCC	This study
rpoCqF	CTAGCTGCTCCTATGTCTCACATC	(2)
rpoCqR	CCAGTCTCTCCTGGATCAACTA	(2)
pdcAqF	AGATATTGCAAACTCAACAAGCTTAAA	This study
pdcAqR	TAATCAAAGCATCGTAAAGCAATGTA	This study
tcdRqF	AGCAAGAAATAACTCAGTAGATGATT	(13)
tcdRqR	TTATTAAATCTGTTTCTCCCTCTTCA	(13)
ilvCqF	AACGGTGTACATGTAATGATAGGTC	This study
ilvCqR	TTTGTAGCTTCTGCTACACTCTTAAC	This study
codYqF	ATTAGGAACATTGGTACTTTCAAGAT	(2)
codYqR	TTGAACTACAGCTTTCTTTCTCATTT	(2)
tcdAqF	GGAGAAGTCAGTGATATTGCTCTTG	This study
tcdAqR	CAGTGGTAGAAGATTCAACTATAGCC	This study
flgBqF	GCAACTAATCTAAGAAGTCAGACAATAGC	This study
flgBqR	AGGCATAGCATCATTTAGTGTTTCTTC	This study
fliCqF	TACAAGTTGGAGCAAGTTATGGAAC	This study
fliCqR	GTTGTTATACCAGCTGAAGCCATTA	This study
OSD107	AAGAGAGATAACTGTTGAAAGATGAGAC	(14)
OSD108	CTTCCATAACCTAAAACTGCAAC	(14)
pdcAemsaF	TGGACAATTTGTCAAGATTAGATCC	This study
pdcAemsaR	CAACAAACTTTTCCTTCTGGATC	This study
gbpAqF	CAGTGGATTAGCGTATGGACAC	(15)
gbpAqR	GTATTGAATCGCGCCACAGT	(15)

*Restriction sites are underlined. Altered nucleotides for generating point mutations are in bold text.

Figure S1

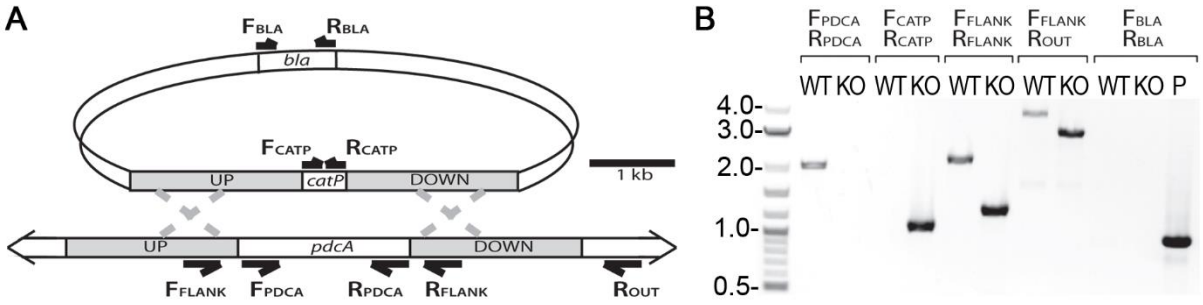

Figure S1. Construction of the *pdcA::catP* mutant. (A) Schematic representation of the double homologous recombination between pMC234::*pdcA*::*catP* and the *C. difficile* 630 chromosome. Regions of homology are in grey. The positions of screening PCR primers are indicated. (B) PCRs were performed with the indicated screening primers using lysates of *C. difficile* 630 (WT) or candidate 630 *pdcA::catP* (KO) as the templates. A control PCR was done with the blaF+blaR primers using purified pMC234::*pdcA::catP* (P).

Figure S2

...tggacaatttgtcaagattagtaccaagttaaaatttaatataagagagagatgtattatgtaatat
taatttttttaaattggtaatactatattgctgtatttttttttatcaattataatttaaaatatgaag
taataaactttctaattaaattaaggtatttagtgtatatgtataagttataagtaaatcttctaaaaag
aataattttaacaatgttgttataaagagcagtaactatggaggagataagtatATGAACAAACATAATT
TTGAAGTTATATTAAATCAACTACAGATTAATATATATGTTACAAATATTCATACTAATGAAATTATTTT
TATGAACAAAAAAATGAAAGAAGAATATAATATTTTAGATCCAGAAGGAAAAGTTTGTTG...

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AATTTTCWGAAAATT     CodY box (L. lactis, B. subtilis)
AATTTTAACAATGTT     putative CodY binding site for pdcA
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Figure S2. Putative CodY binding site upstream of *pdcA*. (A) The sequence of the DNA fragment used in the electrophoretic mobility shift assays is shown (407 bp). Highlighted is the sequence pulled down by CodY in the prior study by Dineen, McBride and Sonenshein (J. Bacteriol, 2010, 192:5350-62). The underlined sequence corresponds to a putative CodY binding site. (B) Alignment of the putative CodY binding site for *pdcA* with the CodY box previously identified for *B. subtilis*, *L. lactis* and *C. difficile*. Asterisks indicate conserved nucleotides, with 4/15 matching the CodY box sequence.

Figure S3.

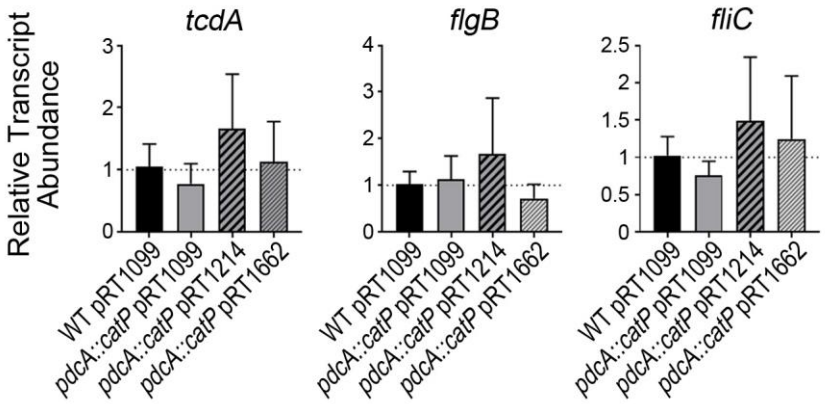

Figure S3. Complementation of toxin and flagellar gene expression during growth in stationary phase. Relative transcript levels of *tcdA*, *flgB*, and *fliC* in wild-type with control vector pRT1099 (black), *pdcA::catP* with control vector (grey), *pdcA::catP* complemented with the wild-type *pdcA* allele in pRT1214 (thick lines), and *pdcA::catP* complemented with the mutant *pdcA*-479A allele in pRT1662 (thin lines) cells during stationary phase. The means and standard deviations from five biological replicates are shown. The data were analyzed by one-way ANOVA and Tukey's multiple comparisons test. No statistically significant differences were found.

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