SUPPLEMENTARY MATERIAL

The *Agrobacterium tumefaciens* CheY-Like Protein ClaR Regulates Biofilm Formation

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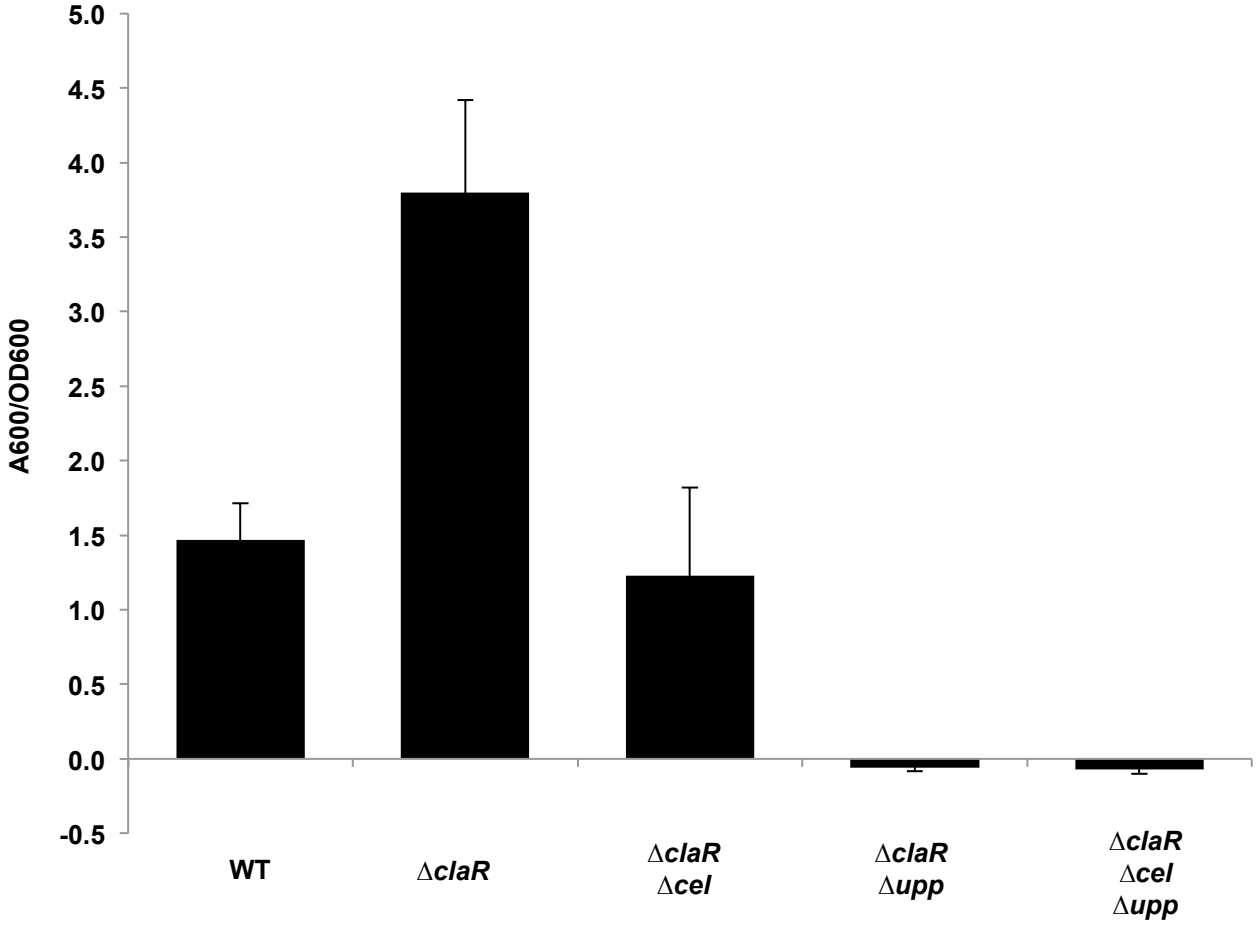
Running title: *Agrobacterium* CheY-type biofilm regulator

S1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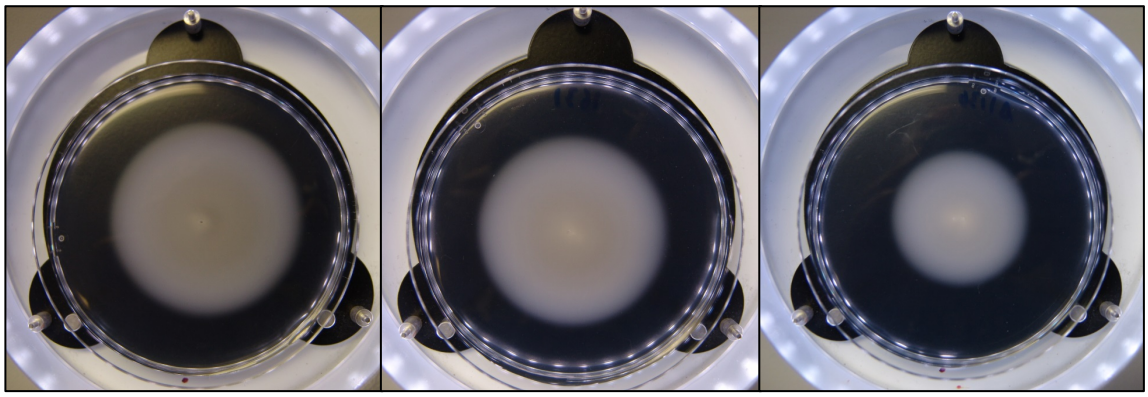
2.0
1.5
1.0
0.5
0.0
-0.5
A600/OD600
WT
ΔclaR
ΔclaR Δcel
ΔclaR Δupp
ΔclaR Δcel Δupp

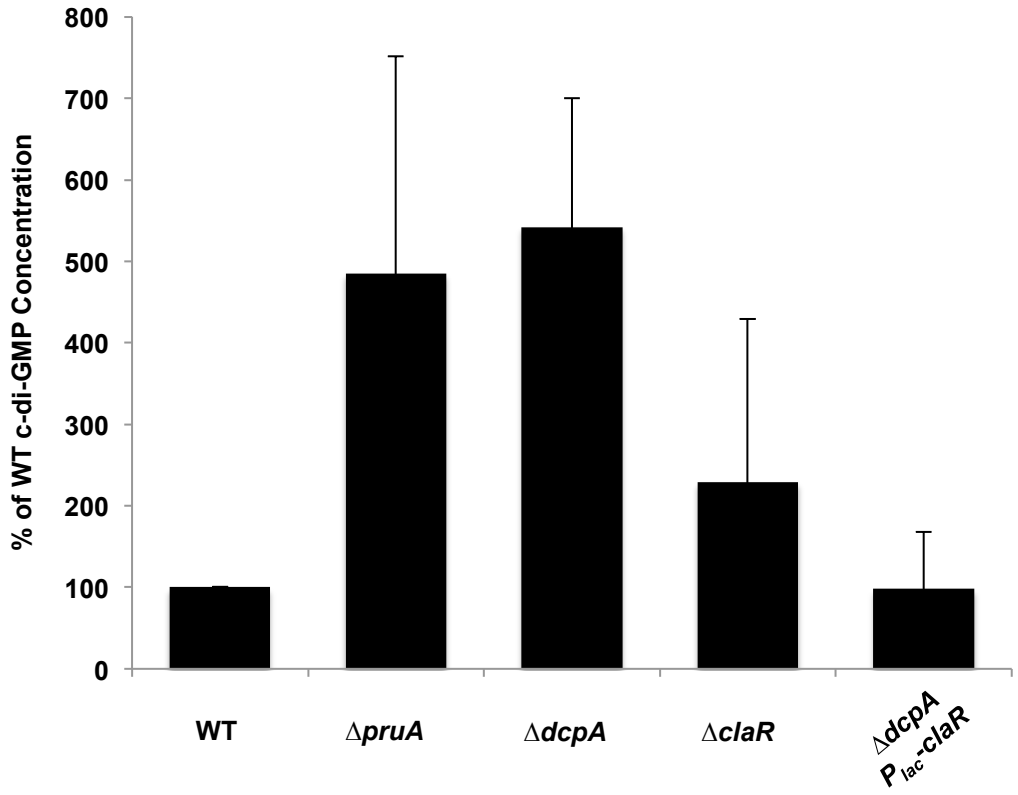
S2a


WT
ΔclaR
ΔpruA

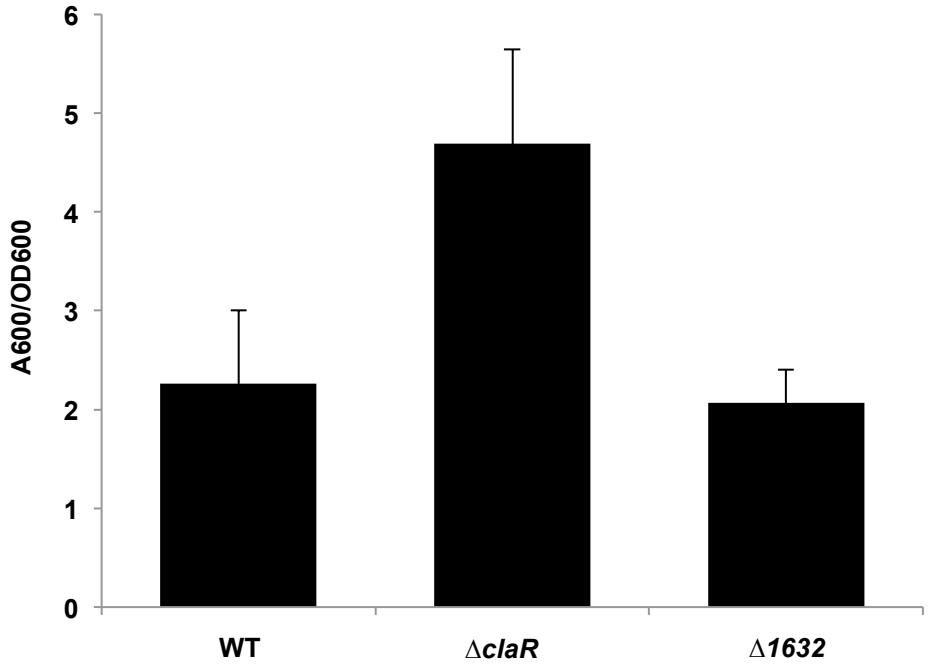
S2b


WT
ΔclaR
ΔpruA

S2c


% of WT c-di-GMP Concentration
800
700
600
500
400
300
200
100
0
WT
ΔpruA
ΔdcpA
ΔclaR
ΔdcpA
Plac-claR

S3



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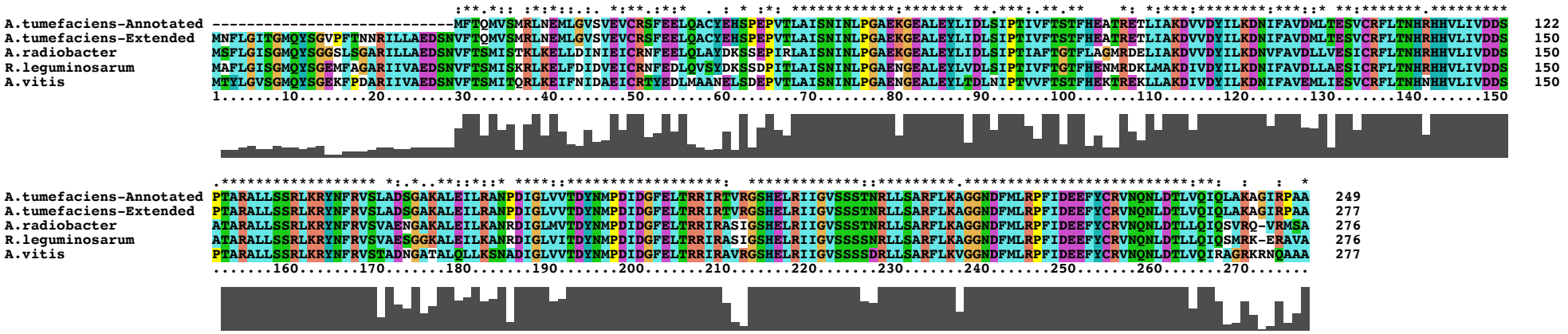
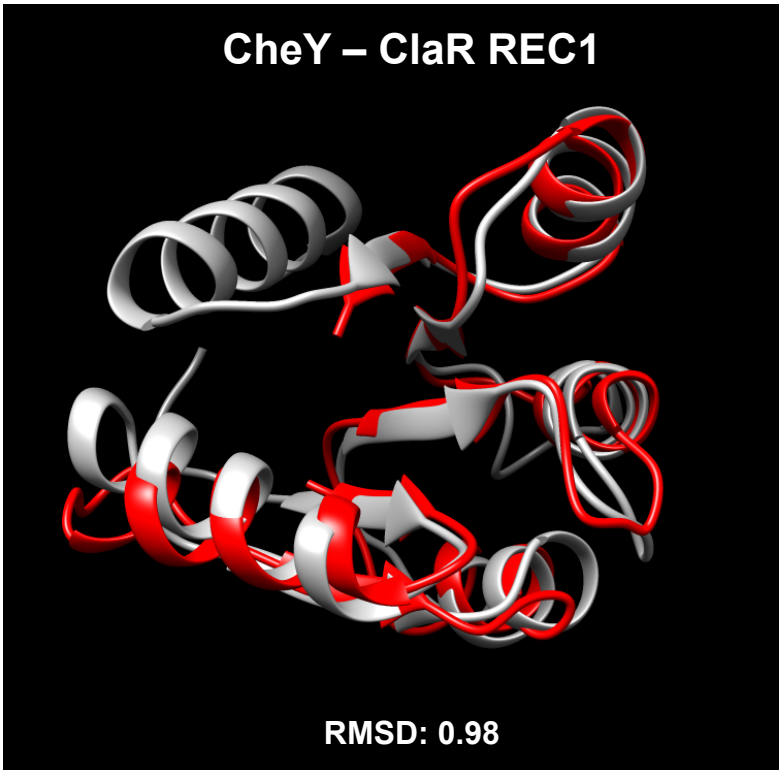
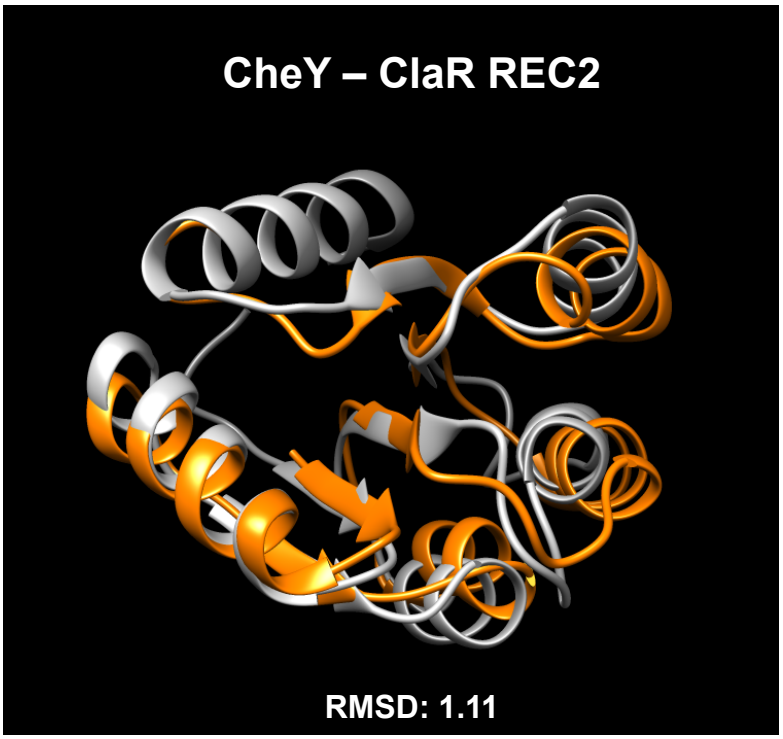

A.tumefaciens-Annotated
A.tumefaciens-Extended
A.radiobacter
R.leguminosarum
A.vitis
1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....110.....120.....130.....140.....150
.....160.....170.....180.....190.....200.....210.....220.....230.....240.....250.....260.....270.....

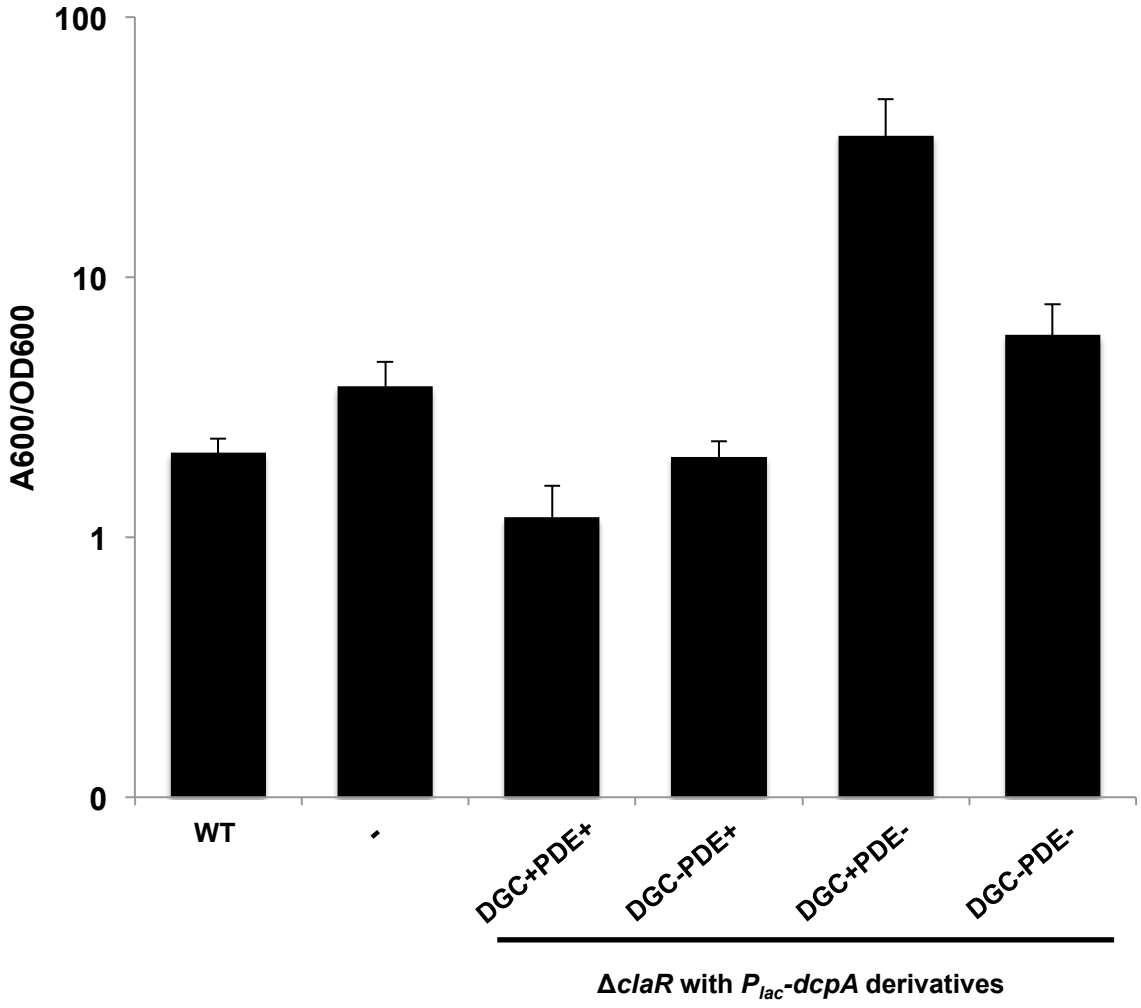
S5a



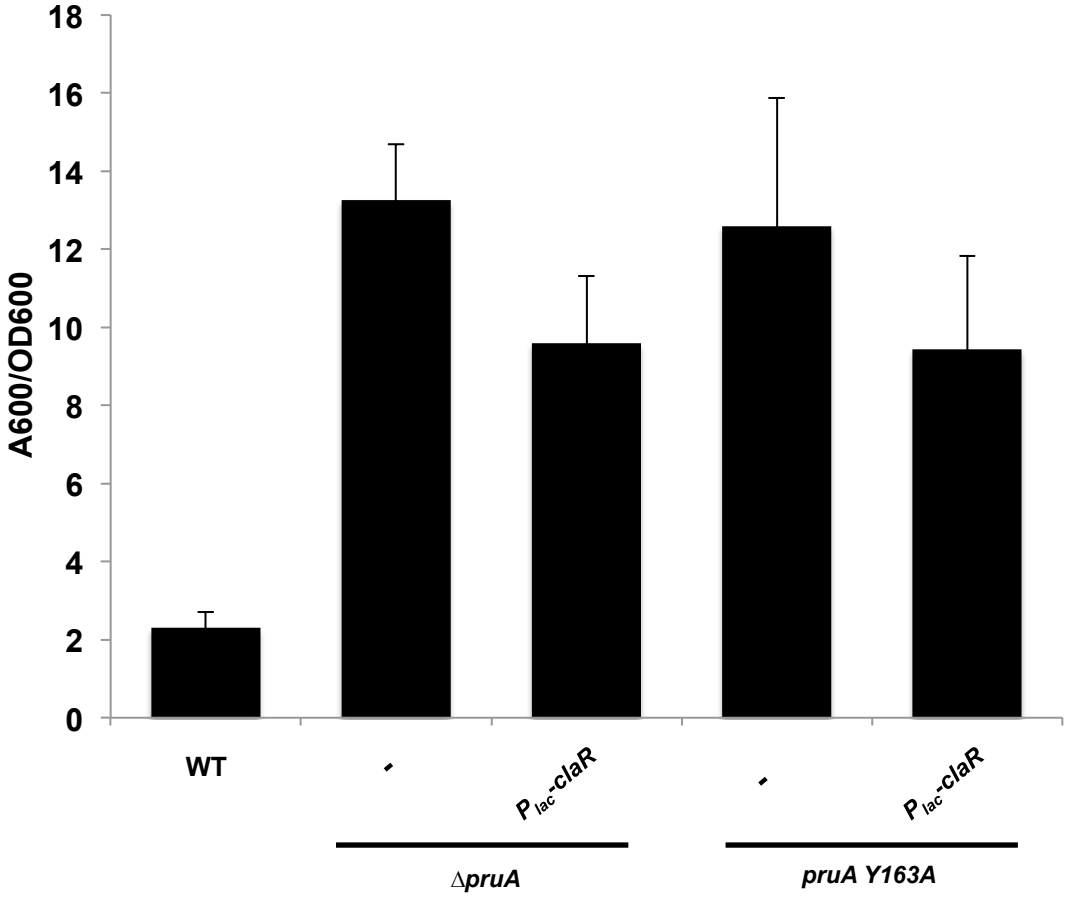
S5b



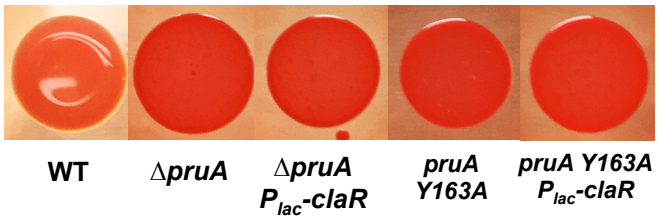
S6


100
10
1
0
A600/OD600
WT
-
DGC+PDE+
DGC-PDE+
DGC+PDE-
DGC-PDE-
ΔclaR with P_lac-dcpA derivatives

S7a


18
16
14
12
10
8
6
4
2
0
A600/OD600
WT
-
P lac-claR
-
P lac-claR
ΔpruA
pruA Y163A

S7b


WT
ΔpruA
ΔpruA Plac-claR
pruA Y163A
pruA Y163A Plac-claR

Supplemental Figure Legends

Figure S1. Polysaccharide dependence of ClaR biofilm phenotypes. 48 hr PVC coverslip biofilms quantified as described in Fig. 2A. Δ*cel* strains are unable to produce cellulose and Δ*upp* strains are unable to produce the UPP. Values are result of two independent biological replicates consisting of three technical replicates each. Error bars: +/- 1 standard deviation.

Figure S2. Additional ClaR phenotypes. (A) *A. tumefaciens* soft agar motility assay. Indicated strains were grown to mid-exponential phase and spotted into ATGN plates containing 0.3% (w v^{-1}) Bacto agar. Plates were dried and placed in room temperature Tupperware container alongside beaker of saturated potassium sulfate. Plates were incubated for 5 days, at which time pictures were taken. (B) Calcofluor staining phenotypes of *A. tumefaciens* colonies. Indicated strains were grown to mid-exponential phase, normalized to an OD_{600} of 0.5 and spotted onto LB plates containing 20 µg/ml calcofluor white (fluorescent white) dye. Spots were dried and photographs taken under UV light exposure in a light cabinet after 48 hours of growth at 28°C. (C) Intracellular c-di-GMP concentrations of *A. tumefaciens* mutants. Indicated strains were grown to early stationary phase, normalized for OD followed by nucleotide extraction. Measurements of c-di-GMP with LC-MS-MS as described in *Materials and Methods.* As with previous experiments, the extended version of ClaR (*claR*-Ext.) was utilized for overexpression studies. Values are the result of two independent biological replicates consisting of three technical replicates each. Error bars: +/- 1 standard deviation.

Figure S3. Atu1632 Doesn't Regulate *A. tumefaciens* biofilm formation. 48 hr PVC coverslip biofilms quantified as described in Fig. 2A. Values are the result of three independent biological replicates consisting of three technical replicates each. Error bars: +/- 1 standard deviation.

Figure S4. Alignment of ClaR Homologs. Alignment of ClaR (Annotated and Extended) to closely related Rhizobial bi-directional best-hit homologs. *A. radiobacter* K84 ACM26930.1 (79% aa identity vs ClaR), *R. leguminosarum* WP_012757872 (79% aa identity vs ClaR), and *A. vitis* WP_015916382.1 (77% aa identity vs ClaR). Alignment performed with ClustalX software (www.clustal.org) using multiple alignment mode.

Figure S5. Secondary structure alignment of predicted ClaR REC domains with experimentally determined CheY structure from *E. coli.* Predicted REC domain Phyre tertiary structures described in Fig. 3 computationally aligned to published *E. coli* crystal structure (3CHY). Alignments performed and visualized using UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) with Needleman-Wunsch alignment algorithm. Coloring as follows: (A) CheY: grey, ClaR REC1: red. (B) CheY: grey, ClaR REC2: orange. Root mean-square deviation (RMSD) values overlaid on alignment images.

Figure S6. DcpA PDE activity can decrease biofilm formation of *claR* mutant. 48 hr PVC coverslip biofilms quantified as described in Fig. 2a. Values are the result of three independent biological replicates consisting of three technical replicates each.

Error bars: +/- 1 SD. DcpA variants containing catalytic site mutations GGDEF->GGDAF (DGC-) and EAL->AAL (PDE-) were examined.

Figure S7: ClaR does not regulate attachment in a PruA catalytic site mutant. (A) 48 hr PVC coverslip biofilms quantified as described in Fig. 2A. Values are the result of three independent biological replicates consisting of three technical replicates each. Error bars: +/- 1 standard deviation. Dash represents strain without plasmid inserted. (B) Congo red colony phenotypes of indicated *A. tumefaciens* strains, as described by Fig. 5B.

Table S1
Strains and plasmids

Strain/plasmid	Relevant features	Reference
E. coli		
DH5α/λpir	*λpir* ; cloning strain	(1)
TOP10 F'	Cloning strain	Invitrogen
S17-1/λpir	*λpir* ; Tra^+ , cloning host	(2)
A. tumefaciens		
C58	Nopaline type strain; pTiC58; pAtC58	(3)
NF001	*pruA Y163A* allelic replacement	(4)
NF003	Δ*pruR*	(4)
NF009	ΔAtu1632	This study
NF011	Δ*claR* Δ*cel*	This study
NF012	Δ*claR* Δ*upp*	This study
NF013	Δ*claR* Δ*cel* Δ*upp*	This study
JX136	Δ*claR*	This study
JX137	Δ*pruA*	(5)
JX138	Δ*dcpA*	(5)
Plasmids		
pGEM-T easy	PCR cloning vector; Ap^R	Promega
pNPTS138	ColE1 suicide plasmid; *sacB;* Km^R	Gift of M. Alley
pSRKGm	Broad host range P_{lac} expression vector; *lacIQ*; Gm^R	(6)
pNF030	pSRKGm carrying P_{lac}-*dcpA E308A*; Gm^R	(4)
pNF031	pSRKGm carrying P_{lac}-*dcpA E431A*; Gm^R	(4)
pNF032	pSRKGm carrying P_{lac}-*dcpA E308A E431A*; Gm^R	(4)
pNF054	pSRKGm carrying P_{lac}-*claR Ext.*; Gm^R	This study
pNF058	pSRKGm carrying P_{lac}-*claR Ext. D193A* Allele; Gm^R	This study
pNF059	pSRKGm carrying P_{lac}-*claR Ext. D193N* Allele; Gm^R	This study
pNF078	pNPTS138 carrying *Atu1632* SOE deletion fragment; Km^R	This study
pJX131	pNPTS138 carrying *claR* SOE deletion fragment; Km^R	This study
pJX148	pSRKGm carrying P_{lac}-*claR Annot.*; Gm^R	This study
pJW168	pSRKGm carrying P_{lac}-*dcpA*; Gm^R	(5)

[a]All *A. tumefaciens* strains are C58 derivatives

Table S2. Oligonucleotides

Primer	Sequence
D193A P1	GTCTCGTCGTGACCGCCTATAATATGCCCG
D193A P2	CGGGCATATTATAGGCGGTCACGACGAGAC
D193N P1	GTCTCGTCGTGACCAACTATAATATGCCCGATATT
D193N P2	CAATATCGGGCATATTATAGTTGGTCACGACGAGA
De1631-P1	(SpeI) actagtCGTTTGCATGAATTGCGTTCGCAG
De1631-P2	*aagcttggtaccgaattc*GCCGGGTTTTCCCGTCAAAGCTGA
De1631-P3	*gaattcggtaccaagctt*CACATTGCTGTCTTCCGCCAG
De1631-P4	(PstI) ctgcagAAGTCGATTGCACTTGCCATGCTC
1632 Del P1	(SpeI) gaactagttaaGTCGCCTCATGGAAGGTGCTG
1632 Del P2	*cagggttctgtaaatgaagaca*CGCGCAT AAGATCATTCCCTC
1632 Del P3	*gagggaatgatcttatgcgcg*TGTCTTCATT TACAGAACCCCTG
1632 Del P4	(NheI) gagctagcgttCGAATTGCGTTTCCACCTTGC
1632 Del P5	GCACATGATGGCGGTGATTGGTG
1632 Del P6	CTTCTGTCGGGAACTGGACG
1631 new 5'	(Nde1) gcgcatATGAATTTCCTGGGCATTAC
Com1631-P1	(SpeI) actagtTCAGCTTTGACGGGAAAACCCGGC
M13-F	CGCCAGGGTTTTCCCAGTCACGAC
M13-R	TCACACAGGAAACAGCTATGAC

Upper case sequence anneals to target sequences; lower case are engineered into oligonucleotides but do not anneal with target sequences in genome

Italicized sequences are complementary among paired oligonucleotides

Engineered restriction endonuclease cleavage sites are indicated in parentheses and highlighted in yellow

Bases altered for site-specific mutagenesis highlighted in red

References

1. **Chiang SL, Rubin EJ.** 2002. Construction of a mariner-based transposon for epitope-tagging and genomic targeting. Gene **296:**179-185.
2. **Kalogeraki VS, Winans SC.** 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene **188:**69-75.
3. **Xu J, Platt TG, Fuqua C.** 2012. Regulatory linkages between flagella and surfactant during swarming behavior: lubricating the flagellar propeller? J Bacteriol **194:**1283-1286.
4. **Feirer N, Xu J, Allen KD, Koestler BJ, Bruger EL, Waters CM, White RH, Fuqua C.** 2015. A Pterin-Dependent Signaling Pathway Regulates a Dual-Function Diguanylate Cyclase-Phosphodiesterase Controlling Surface Attachment in *Agrobacterium tumefaciens*. mBio **6**.
5. **Xu J, Kim J, Koestler BJ, Choi JH, Waters CM, Fuqua C.** 2013. Genetic analysis of *Agrobacterium tumefaciens* unipolar polysaccharide production reveals complex integrated control of the motile-to-sessile switch. Mol Microbiol.
6. **Khan SR, Gaines J, Roop RM, 2nd, Farrand SK.** 2008. Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. Appl Environ Microbiol **74:**5053-5062.