Supplementary Information

Identification of small molecules that interfere with c-di-GMP signaling and induce dispersal of *Pseudomonas aeruginosa* biofilms

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Supplementary Tables

Supplementary Table 1. Strain list.

Strains	Description	Reference
E.coli :		
DH5-α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)*U169, hsdR17(r_k^- m_k^+), λ^-	Lab collection
HB101	*rec*A *thi pro leu hsd*RM$^+$, Sm^R	Kessler et al. 1992
RK600	HB101 carrying pRK600	Kessler et. al. 1992
S17-1 λ*pir*	Str^R, Tet^R, F- RP4-2-Tc::Mu *aph*A::Tn7 *rec*A λpir lysogen	Lab collection
S17-1	*recA pro* (RP4-2Tet::Mu Kan::Tn*7*)	Simon et al. 1983
AR3110	Cellulose producing strain	Serra et al. 2013
P. aeruginosa:		
PAO1	*Pseudomonas aeruginosa* reference strain	Stover et al. 2000
PAO1::*Tn7-gfp*	PAO1 chromosomally tagged with *gfp*	Andersen et al. 2021
Δ*wspF*	*wspF* deletion mutant of PAO1	Rybtke et al. 2012
Δ*bifA*	*bifA* deletion mutant of PAO1	This study
Δ*bifA*Δ*wspF*	*wspF, bifA* double deletion mutant of PAO1	This study
ΔwspFΔpelΔpsl	*wspF*, *pel*, *psl* triple deletion mutant of PAO1	Rybtke et al. 2012
ΔwspFΔpelΔpsl/pCdrA-gfp	*ΔwspFΔpelΔpsl* carrying plasmid pCdrA-gfp	Rybtke et al. 2012
ΔbifAΔwspFΔpelΔpsl	*bifA, wspF*, *pel*, *psl* quadruple deletion mutant of PAO1	This study
ΔbifAΔwspFΔpelΔpsl/pCdrA-gfp	*ΔbifAΔwspFΔpelΔpsl* carrying plasmid pCdrA-gfp	This study
ΔbdlAΔwspFΔpelΔpsl/pCdrA-gfp	*ΔbdlAΔwspFΔpelΔpsl* carrying plasmid pCdrA-gfp	This study
ΔsiaDΔwspFΔpelΔpsl/pCdrA-gfp	*ΔsiaDΔwspFΔpelΔpsl* carrying plasmid pCdrA-gfp	This study
ΔwspFΔpelΔpsl/pYhjH^G	*ΔwspFΔpelΔpsl* carrying plasmid pYhjH^G	This study
Δ*bifA*Δ*wspF* P_{BAD}-*bifA*	Δ*bifA*Δ*wspF* with chromosomal insertion of *ara*C-P_{BAD}-*bif*A	This study
PAO1(Iglewski)	PAO1 with reduced production of C4-HSL (Köhler et al. 2001)	Obtained from B. Iglewski (University of Rochester Medical Center, NY, USA)
MPAO1	*P. aeruginosa* PAO1 from Dr. Manoils laboratory.	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW1520)	PA0285-H01::ISlacZ/hah	Jacobs et al. 2003, Held et

		al. 2012
MPAO1(PW4747)	PA2200-F11::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW5718)	arr(PA2818)-A01::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW7454)	PA3825-G08::ISphoA/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW5308)	PA2567-G11::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW9918)	PA5295-G02::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW7948)	PA4108-B01::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW7674)	rocR(PA3947)-E06::ISphoA/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW4043)	mucR(PA1727)-C01::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW8371)	bifA(PA4367)-D09::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW8754)	morA(PA4601)-C02::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW2569)	rdbA(PA0861)-F02::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW6567)	nbdA(PA3311)-B12::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW9424)	dipA(PA5017)-A01::ISphoA/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW2060)	PA0575-A03::ISphoA/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW3134)	PA1181-H12::ISphoA/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW3602)	PA1433-B09::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW4568)	PA2072-B02::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW6465)	PA3258-D03::ISphoA/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW9346)	fimX(PA4959)-F01::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW10193)	PA5442-C04::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW4665)	PA2133-B04::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012
MPAO1(PW2283)	PA0707-E04::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012.
MPAO1(PW5317)	PA2572-D10::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012.
MPAO1(PW9032)	PA4781-E02::ISlacZ/hah	Jacobs et al. 2003, Held et al. 2012.

Supplementary Table 2. Plasmid list.

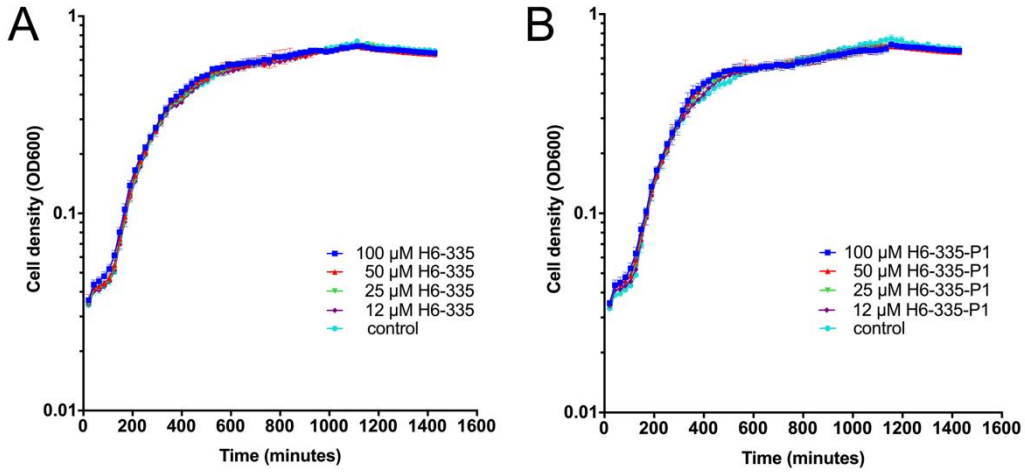
Plasmid	Description	Reference
pK18GT-bifA-del	*bif*A deletion vector, Gm^R	An et al. 2010
pJBAMG13	*ara*C-P_{BAD}-*bif*A fusion of pJBAMG10 inserted into the BP clonase sites of pminiCTX2T2.1-GW	Andersen et al. 2021
pBT20	Transposon delivery vector	Kulasekara 2014
pJN105	Arabinose inducible expression vector	Newman & Fuqua 1999
pJN105::bifA	Plasmid for arabinose induction of *bifA*. Also termed pJBAMG10.	Andersen et al. 2021
pCdrA-gfp	pUCP22Not-P_{cdrA}-*gfp*(mut3)-T_0-T_1, Amp^R, Gm^R	Rybtke et al. 2012
pFlp2	Source of Flp2 recombinase, Amp^R	Hoang et al. 1998
pRK600	Mobilization plasmid, Cm^R	Kessler et al. 1992
pQE30	Expression vector of N-terminally His-tagged proteins Amp^R	Qiagen
pQE30::bifA180	Plasmid for IPTG-inducible expression of BifA180. A *bifA*180 fragment was PCR amplified from *P. aeruginosa* DNA with primers Bif180-*Sac*I and Bif180-*Hind*III, and cloned in the *Sac*I and *Hind*III site of pQE30.	
pYjhH	*yhjH* gene of *E. coli* cloned in pBBR1MCS3, Tet^R, Cm^R	Gjermansen et al. 2006
pBBR1MCS5	Broad host range plasmid, Gm^R, Cm^R	Kovach et al. 1995
$pYhjH^G$	*yhjH* expression cassette of pYhjH cloned into plasmid pBBR1MCS5, Gm^R	This study

Supplementary Table 3. Primer list

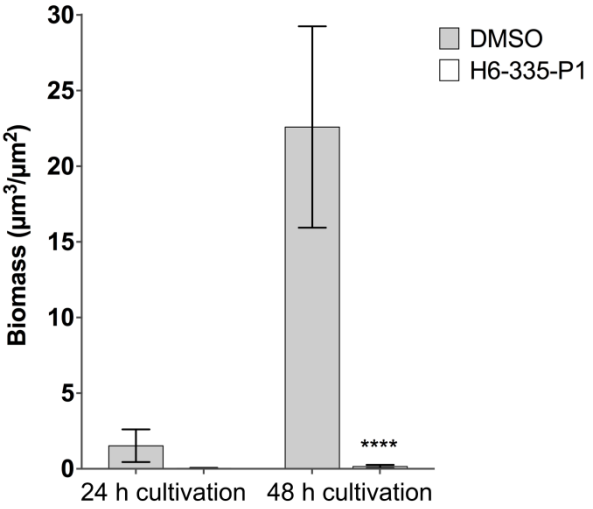
Primers	Description	References
rpoD fwd	5'-ACAAGATCCGCAAGGTACTGAAG-3'	Jakobsen et al. 2012
rpoD rev	5'-CGCCCAGGTGCGAATC-3'	Jakobsen et al. 2012
rpoS fwd	5'-CTCCCCGGGCAACTCCAAAAG-3'	This study
rpos rev	5'-CGATCATCCGCTTCCGACCAG-3'	This study
oprL fwd	5'-ATGGAAATGCTGAAATTCGGC-3'	This study
oprl rev	5'-ACCTTCACCGGAAGCATCG-3'	This study
bifA fwd	5'-CATCTTCATCTCCGGCATTCTC-3'	This study
bifA rev	5'-TTGGTCAGCATCCAGTGGTAGA-3'	This study
cdrA fwd	5'-CAACAGTCAGTTCAACGACCTC-3'	This study
cdrA rev	5'-AACGCTGGCTGAAATACTCG-3'	This study
*bif*A-D-Up-F-*Eco*RI	5'-GGAATTCTGCTGACCTGCGACGTCTGGGAAC-3'	An et al. 2010
*bif*A-D-Dn-R-*Hind*III	5'-CCCAAGCTTGTGCGGTGATCCGTGAATGGAAGG-3'	An et al. 2010
Pser-up	5'-CGAGTGGTTTAAGGCAACGGTCTTGA-3'	Hoang et al. 2000
Pser-down	5'-AGTTCGGCCTGGTGGAACAACTCG-3'	Hoang et al. 2000

TnMseq	5’-CACCCAGCTTTCTTGTACAC-3’	Rybtke et al. 2015
Rnd1-TnM	5’-GTGAGCGGATAACAATTTCACACAG-3’	Rybtke et al. 2015
Rnd1-pP1	5’-GGCCACGCGTCGACTAGTCANNNNNNNNNNGAT AT-3’	Rybtke et al. 2015
Rnd1-pP2	5’-GGCCACGCGTCGACTAGTCANNNNNNNNNNACG CC-3’	Rybtke et al. 2015
Rnd2-TnM	5’- ACAGGAAACAGGACTCTAGAGG-3’ ??	Rybtke et al. 2015
Rnd2-Pp	5’- GGCCACGCGTCGACTAGTAC-3’ ??	Rybtke et al. 2015
BifA180-*Sac*I	5’- GGCGGAGCTCCACTGGATGCTGACCAAGCC	This study
BifA180-*Hind*III	5’- GGCGAAGCTTTCAGGGCCGTTCGCTGCTGGTGG	This study

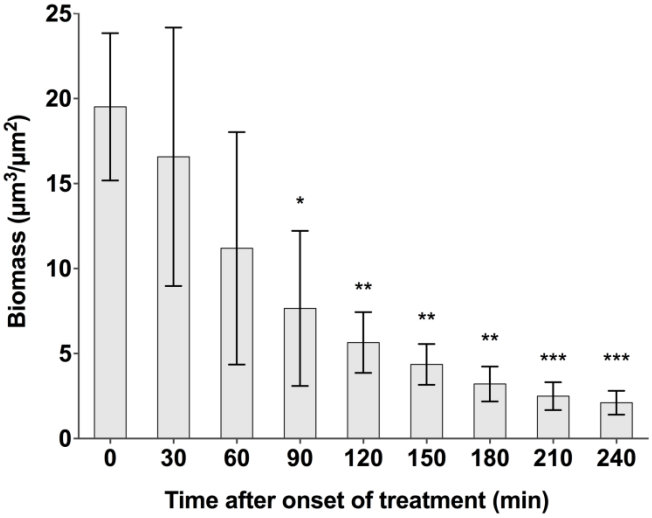
Supplementary Figures



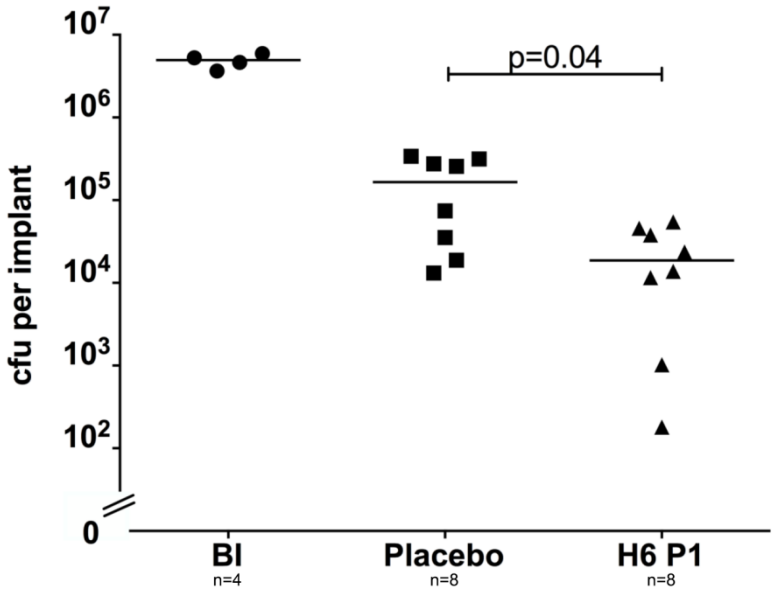
Supplementary Figure 1. Effects of H6-335 **(A)** and H6-335-P1 **(B)** on the growth of the c-di-GMP monitor strain *P. aeruginosa* Δ*wspF*Δ*pel*Δ*psl*/pCdrA-gf*p.* Bacteria where grown in the wells of microtiter plates in the presence of various concentrations of H6-335 or H6-335-P1. Cell density (OD_{600}) was measured every 20 minutes for 24 hours. Cell densities are plotted as a function of time and H6-335 or H6-335-P1 concentration. Mean and standard deviation (bars) of 3 biological replicates (n=3) are shown.



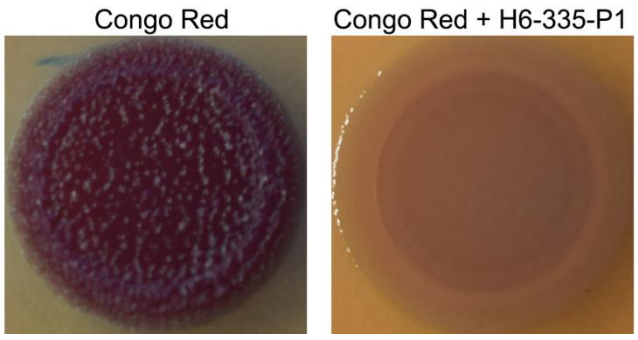
Supplementary Figure 2. H6-335-P1-mediated inhibition of *P. aeruginosa* biofilm formation in flow-cells. *P. aeruginosa* was cultivated in flow-cells perfused with growth medium with or without 25 µM H6-336-P1. CLSM micrographs of the adherent bacteria were acquired after 24 and 48 hours of cultivation, and the amount of bacterial biomass in the flow-cells was quantified by the use of COMSTAT image analysis (Heydorn et al., 2000). Average biomass calculated from three independent biofilms is shown. Error bars indicate standard deviations. One-way ANOVA analysis was used to calculate significance values (****, p<0.0001)



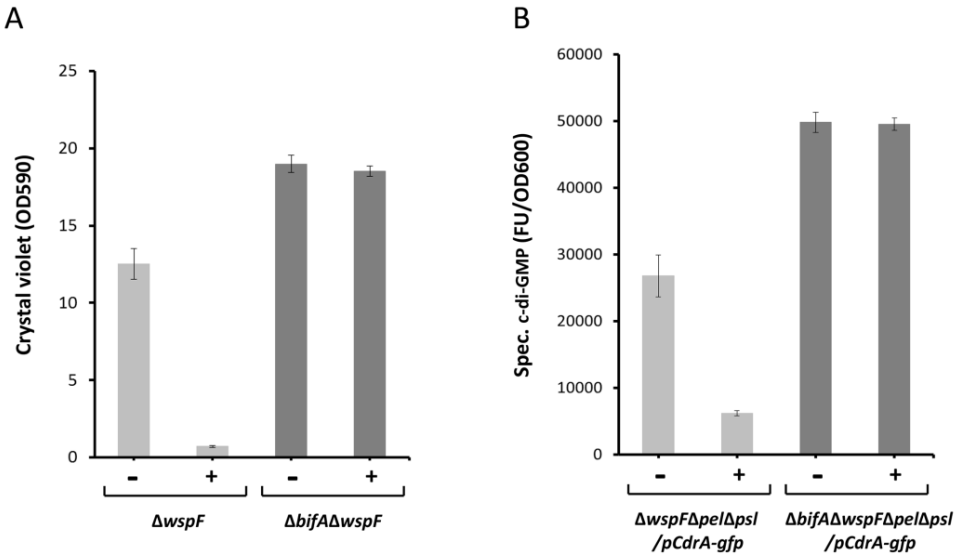
Supplementary Figure 3. H6-335-P1 mediated dispersal of *P. aeruginosa* biofilms in flow-cells. *P. aeruginosa* biofilms were cultivated in flow-cells irrigated with growth medium supplemented with 0.025% DMSO. After 48 hours of cultivation the flow-through medium was shifted so that it contained 0.025% DMSO and 25 µM H6-335-P1. CLSM micrographs were acquired immediately before the introduction of H6-336-P1 and at 30 minutes intervals after the introduction of the compound. The amount of bacterial biomass in the flow-cells was quantified by the use of COMSTAT image analysis (Heydorn et al., 2000). Average biomass calculated from three independent biofilms is shown. Error bars indicate standard deviations. One-way ANOVA analysis was used to calculate significance values compared to time zero (*, p<0.05; **, p<0.01; ***, p<0.001).



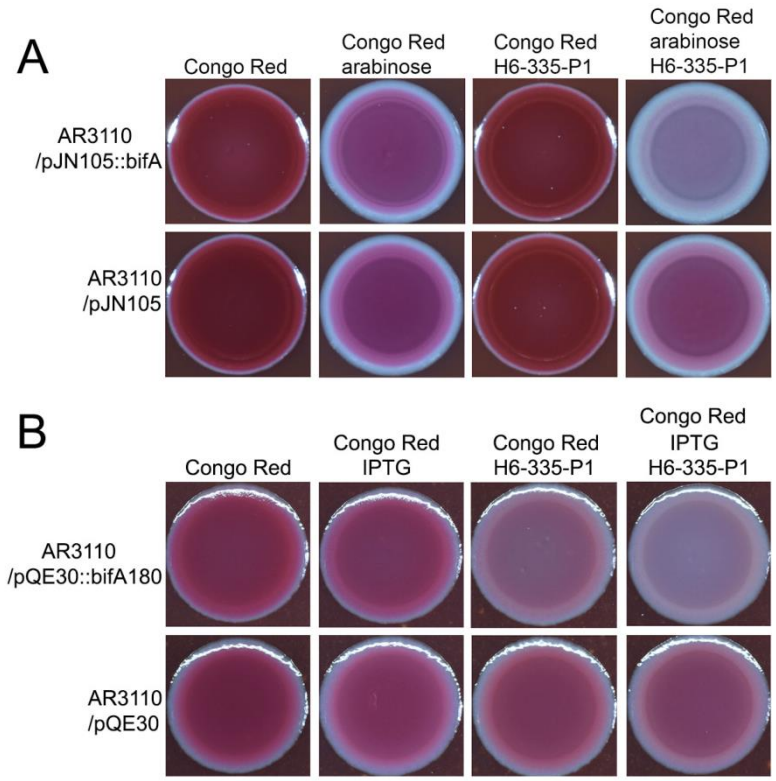
Supplementary Figure 4. The effect of treatment of *P. aeruginosa* biofilm infections with H6-335-P1. Silicone implants were incubated with *P. aeruginosa* for 20 hours for bacterial adhesion. At time zero, mice had the implants inserted in the peritoneal cavity. At 24 hours and 26 hours post-insertion of the implants (PI), the mice were treated with either H6-335-P1 (H6 P1) (6 μM corresponding to 1 μg compound per gram bodyweight) or vehicle (as placebo). At 28 hours PI the mice were euthanized and the CFU per implant was determined. Significance level is based on Mann-Whitney *U* test (analysis of non-parametric data). BI: Bacterial count before insertion of implants.



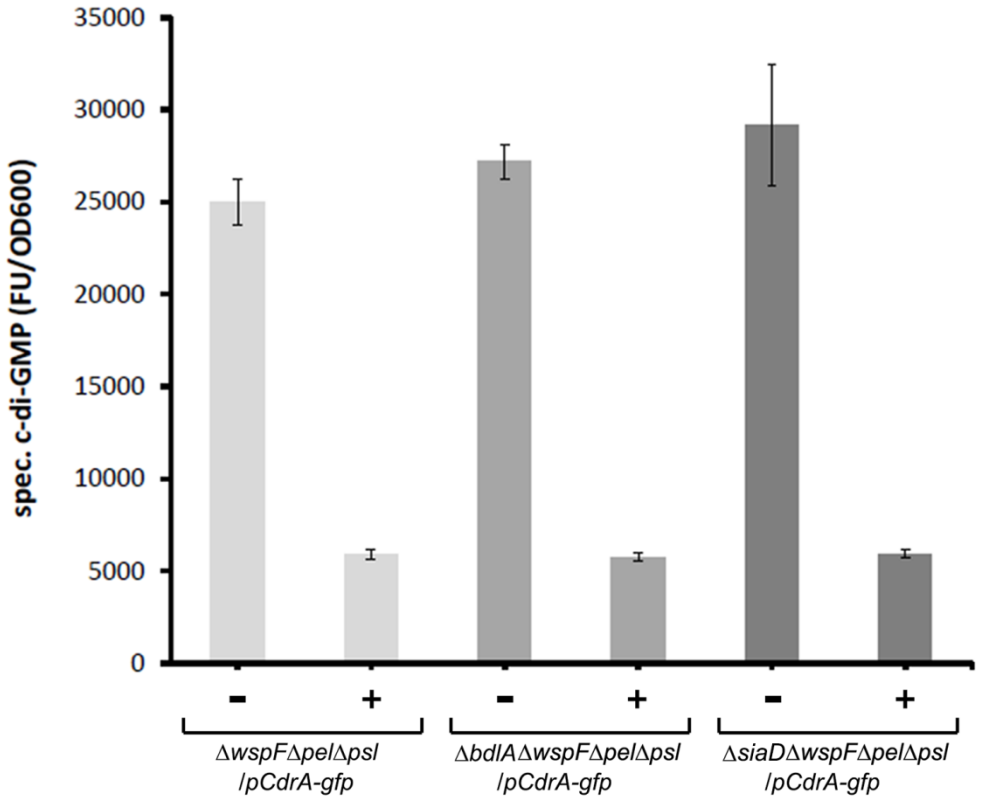
Supplementary Figure 5. Morphology of *P. aeruginosa wspF* macrocolonies on agar plates supplemented with Congo Red or Congo Red and H6-335-P1. Macrocolonies were grown from 5 μl overnight culture spotted on the agar plates. Photographs of the macrocolonies were acquired after 48 hours incubation at 30°C. Note that H6-335-P1 is yellow and colors the agar plates that are supplemented with this compound.



Supplementary Figure 6. (**A**) Amount of biofilm formed in microtiter trays by *P. aeruginosa ΔwspF* and *ΔbifAΔwspF* strains in the absence (-) or presence (+) of 100 μM H6-335-P1. Mean and standard deviation (bars) of three replicates are shown. (**B**) Fluorescence readout from *P. aeruginosa ΔwspFΔpelΔpsl*/pCdrA-gfp and *ΔbifAΔwspFΔpelΔpsl*/pCdrA-gfp cultures grown in the absence (-) or presence (+) of 100 μM H6-335-P1. Sampling was done after 17 hours of growth (early stationary phase). Mean and standard deviation (bars) of three replicates are shown.



Supplementary Figure 7. Morphology of *E. coli* macrocolonies on agar plates. (**A**) Macrocolonies were grown from 5 µl overnight culture of *E. coli* AR3110/pJN105::bifA or *E. coli* AR3110/pJN105 (vector control) spotted on no-salt LB agar plates supplemented with respectively Congo Red (160 µg/ml), Congo Red and arabinose (0.2%), Congo Red and H6-335-P1 (100 µM), or Congo Red, arabinose and H6-335-P1. Photographs of the macrocolonies were acquired after 27 hours incubation at 28°C. (**B**) Macrocolonies were grown from 5 µl overnight culture of *E. coli* AR3110/pQE30::bifA180 or *E. coli* AR3110/pQE30 (vector control) spotted on agar plates supplemented with respectively Congo Red, Congo Red and IPTG (0.4 mM), Congo Red and H6-335-P1, or Congo Red, IPTG and H6-335-P1. Photographs of the macrocolonies were acquired after 24 hours incubation at 28°C.



Supplementary Figure 8. Fluorescence readout from *P. aeruginosa ΔwspFΔpelΔpsl*/pCdrA-gfp, *ΔbdlAΔwspFΔpelΔpsl* /pCdrA-gfp and *ΔsiaDΔwspFΔpelΔpsl*/pCdrA-gfp cultures grown in the absence (-) or presence (+) of 100 μM H6-335-P1. Sampling was done after 17 hours of growth (early stationary phase). Mean and standard deviation (bars) of three replicates are shown.

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