Supporting Information

Deng & Schmid et al., "~~The fatty acid signal~~ receptor RpfR links quorum~~-~~sensing ~~-~~with regulation of virulence through ~~cyclic-di-GMP~~ turnover"

Experimental methods:

Construction of Mutants and Complementation of Strains. *B. cenocepacia* H111 was used as the parental strain to generate the in-frame deletion mutants of $rpfF_{Bc}$ and *rpfR*, respectively, following the methods described previously (16). The primers to generate upstream and downstream regions flanking $rpfF_{Bc}$ and *rpfR* are listed in SI Appendix, Table S3. For complementation analysis, the coding regions of $RpfF_{Bc}$, RpfR and the relevant domains of RpfR were amplified by PCR using the primers listed in SI Appendix, Table S3, and cloned under the control of the S7 ribosomal protein promoter in plasmid vector pMSL7 or under the control of *lac* promoter in the vector pLAFR3. The resulting constructs were conjugated into *B. cenocepacia* H111 deletion mutants using tri-parental mating with pRK2013 as the mobilizing plasmid.

Determination of Intracellular Cyclic-Di-GMP Level. *B. cenocepacia* strain H111 and its derivatives were grown in 1 liter of NYG medium at 37^{o}C for 24 h with shaking at 200 rpm. Intracellular cyclic-di-GMP molecules were isolated as described previously (31). The samples were filtered using a 0.2 µm pore size cellulose-acetate filter, and 20 µl of each sample was detected at 252 nm on a Waters LC chromatographic system on a reverse-phase column (Phenomenex Luna, 5 µm C18, 250 by 4.60 mm) and eluted with an isocratic mobile phase (150 mM NaH_2PO_4, pH 5.2, containing 2% acetonitrile, v/v) at 1 ml min^{-1}. Synthetic cyclic-di-GMP (BioLog) was used as a standard.

Biofilm Formation, Swarming Motility and Proteolytic Activity Assays. Biofilm formation in 96-well polypropylene microtiter dishes was assayed essentially as described previously by Huber et al (2001) (32). Swarming motility was determined on semi-solid agar (0.5%). Bacteria were inoculated into the center of plates containing 0.8% tryptone, 0.5% glucose, and 0.5% agar. The plates were incubated at 37°C for 24 h before the diameter of the colony was measured. Protease assay was performed following the previously described method

(33). Protease activity was obtained after normalization of absorbance against corresponding cell density.

Protein Expression and Purification. The coding region of *rpfR* was amplified with the primers listed in Table S3 and fused to the expression vector pGEX-6p-1 (Amersham). The fusion gene construct was transformed into the *E. coli* strain BL21. Affinity purification of GST-RpfR fusion proteins was performed following the method described previously (34). Fusion protein cleavage with PreScission Protease (GE Healthcare; 2 units/100 µl of bound proteins) was conducted at 4°C overnight. The cleaved fusion proteins were eluted and analyzed by SDS-PAGE.

Site-Directed Mutagenesis of pBBR-*rpfR*. Point mutations in pBBR-*rpfR* were generated on the basis of the QuikChange site directed mutagenesis system (Agilent). Briefly, to introduce the desired point mutations, the plasmid was amplified with *PfuTurbo* DNA Polymerase (Agilent) using the primer pairs AAL-fw/AAL-rev or GGAAF-fw/GGAAF-rev that contain the desired nucleotide substitution. The reaction mix was then digested with *Dpn*I and transformed into *E. coli* XL1blue. Mutations were confirmed by sequencing.

CD Spectroscopy and ITC. Far-UV circular dichroism (CD) analysis of RpfR was carried out on a JASCO J-810 spectropolarimeter as previously described (35). RpfR and BDSF solutions were mixed at room temperature for 1 h at a final concentration of 20 µM and 200 µM, respectively. The isothermal titration calorimetry (ITC) measurements were obtained using a VP-ITC ITC microcalorimeter following the manufacturer's protocol (MicroCal, Northampton, MA). In brief, titrations began with one injection of 2 µl of BDSF solution into the sample cell containing 1.4 ml of RpfR solution (20 µM) in the VP-ITC microcalorimeter. The volume of BDSF injection was changed to 10 µl in the subsequent twenty-eight injections. The heat changes accompanying injections were recorded. The titration

experiment was repeated at least twice, and the data were calibrated with a buffer control and fitted with the one-site model to determine the binding constant (*Ka*) using the MicroCal ORIGIN version 7.0 software.

Enzyme Activity Analysis. The enzyme activity of RpfR was determined following the methods previously described (34). BDSF and RpfR in PBS buffer were mixed and incubated at room temperature for 1 h. An equal volume of PBS buffer was used as control. Cyclic-di-GMP was dissolved in TME buffer (Tris-HCl, 60 mM; $MgCl_2$, 10 mM; and EDTA, 1 mM; pH 7.5) and added to the mixture at a final concentration of 250 μM, while the final concentrations of BDSF and RpfR were 50 μM and 2 μM, respectively. The reaction mixture was kept at 37°C, and aliquots (50 μl) of samples were taken at various time points as indicated. The reaction was stopped by placing the sample tube in boiling water for 5 min. Cyclic-di-GMP level was measured by HPLC as described above.

Table S1. Conservation of RpfR and $RpfF_{Bc}$ in various bacterial species

Bacteria	Strain	RpfR homologue Accession No.	RpfR homologue Identity (%)	$RpfF_{Bc}$ homologue Accession No.	$RpfF_{Bc}$ homologue Identity (%)
Burkholderia					
B. cenocepacia	J2315	CAR54438	100	CAR54439	100
B. cepacia	383	ABB12684	96	ABB12683	95
B. multivorans	ATCC17616	ABX18792	93	ABX18791	94
B. dolosa	AUO158	EAY71441	92	EAY71442	97
B. ambifaria	MEX-5	EDT41907	92	EDT41908	95
B. vietnamiensis	G4	ABO57013	91	ABO57014	95
B. ubonensis	Bu	ZP_02382568	86	ZP_02382569	90
B. phymatum	STM815	ACC74359	66	ACC74358	74
B. xenovorans	LB400	ABE34804	66	ABE34805	71
B. graminis	C4D1M	EDT13013	66	EDT13012	72
B. phytofirmans	PsJN	ACD19812	66	ACD19813	72
Achromobacter					
A. xylosoxidans	A8	ADP15809	63	ADP15810	70
A. piechaudii	ATCC43553	EFF75758	63	EFF75759	68
A. sp.	SY8	EHK66463	62	EHK66462	68
Yersinia					
Y. mollaretii	ATCC43969	EEQ10338	61	EEQ10337	67
Y. aldovae	ATCC35236	EEP94463	61	EEP94464	67
Y. intermedia	ATCC29909	EEQ17658	61	EEQ17659	68
Y. ruckeri	ATCC29473	EEP98557	61	EEP98558	67
Y. enterocolitica subsp. palearctica	105.5R(r)	ADZ40552	61	ADZ40551	67
Y. bercovieri	ATCC43970	EEQ07113	61	EFQ07114	69
Y. rohdei	ATCC43380	EEQ03158	61	EEQ03157	67
Y. kristensenii	ATCC33638	EEP90234	61	EEP90233	68
Y. frederiksenii	ATCC33641	EEQ13950	60	EEQ13949	67
Y. enterocolitica subsp enterocolitica	8081	CAL10197	60	CAL10196	67
Serratia					
S. odorifera	4Rx13	EFA14792	61	EFA14793	69
S. sp.	AS9	AEF47930	61	AEF47931	69
S. proteamaculans	568	ABV43835	60	ABV43836	70
Enterobacter					
E. asburiae	LF7a	AEN65083	57	AEN65084	63
E. cancerogenus	ATCC35316	EFC56872	57	EFC56873	63

E. cloacae sp. *cloacae*	ATCC 13047	YP_003612261	57	YP_003612260	63
E. mori	LMG25706	ZP_09038026	56	ZP_09038027	63
E. sp.	638	ABP60856	56	ABP60857	63
Pantoea					
P. sp.	At-9b	ADU69377	57	ADU69378	65
P. ananatis	LMG5342	CCF09523	56	CCF09522	64
Cronobacter					
C. turicensis	Z3032	CBA31265	56	CBA31267	61
C. sakazakii	ATCC BAA-894	YP_001437678	56	YP_001437677	61
Others					
Rahnella sp.	Y9602	ADW72158	58	ADW72157	70
Erwinia billingiae	Eb661	CAX59882	57	CAX59883	64
Yokenella regensburgei	ATCC43003	EHM47523	56	EHM47522	62

Table S2. Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotypes and/or characteristics	Reference or source
B. cenocepacia		
H111	Wild type strain, Genomovars III of the *B. cepacia* complex	(Huber *et al.*, 2001)
H111(GGDEF)	Wild type strain harboring the expression construct pLAFR3-GGDEF	This study
H111(EAL)	Wild type strain harboring the expression construct pLAFR3-EAL	This study
ΔrpfF$_{Bc}$	BDSF-minus mutant derived from H111 with *rpfF$_{Bc}$* being deleted	This study
ΔrpfR	Deletion mutant with *rpfR* being deleted	This study
ΔrpfF$_{Bc}$ (rpfF$_{Bc}$)	Mutant ΔrpfF$_{Bc}$ harboring the expression construct pMLS7-rpfF$_{Bc}$	This study
ΔrpfR (rpfR)	Mutant ΔrpfR harboring the expression construct pMLS7-rpfR	This study
ΔrpfF$_{Bc}$ (rpfR)	Mutant ΔrpfF$_{Bc}$ harboring the expression construct pLAFR3-rpfR	This study
ΔrpfF$_{Bc}$(GGDEF-EAL)	Mutant ΔrpfF$_{Bc}$ harboring the expression construct pLAFR3-GGDEF-EAL	This study
ΔrpfF$_{Bc}$ (GGDEF)	Mutant ΔrpfF$_{Bc}$ harboring the expression construct pLAFR3-GGDEF	This study
ΔrpfF$_{Bc}$ (EAL)	Mutant ΔrpfF$_{Bc}$ harboring the expression construct pLAFR3-EAL	This study
ΔrpfF$_{Bc}$ (rocR)	Mutant ΔrpfF$_{Bc}$ harboring the expression construct pMLS7-rocR	This study
ΔrpfR (rocR)	Mutant ΔrpfR harboring the expression construct pMLS7-rocR	This study
ΔrpfRBCAM0227	Mutant with *rpfR* and *BCAM0227* being deleted and interrupted by pEX18Gm, respectively	This study
rpfR	Insertional mutant with *rpfR* being interrupted by pEX18Gm	This study
rpfR(rpfR)	Mutant rpfR harboring the expression construct pBBR-rpfR	This study
rpfR(rpfR$_{AAL}$)	Mutant rpfR harboring the expression construct pBBR-rpfR$_{AAL}$	This study
rpfR(rpfR$_{GGAAF}$)	Mutant rpfR harboring the expression construct pBBR-rpfR$_{GGAAF}$	This study
BCAM0227	Insertional mutant with *Bcam0227* being interrupted by pEX18Gm	This study
E. coli		
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir*	Laboratory collection
BL21	F$^-$*ompT hsdS* ($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal* (DE3) *endA*	Stratagene
OP50	A uracil auxotroph strain as a food source for *C. elegans*	(Brenner, 1974)
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI^{q}ZΔM15* Tn*10* Tet^r	Stratagene

Plasmid		
pMLS7-rpfF$_{Bc}$	pMLS7 containing *rpfF$_{Bc}$*	This study
pMLS7-rpfR	pMLS7 containing *rpfR*	This study
pLAFR3-rpfR	pLAFR3 containing *rpfR*	This study
pEX18Gm	pUC18 MCS, *sacB*$^{+}$; gene replacement vector; Gm^{r}	(Hoang *et al.*, 1998)
pEX-rpfR	pEX18 containing an internal fragment of *rpfR*	This study
pEX-0227	pEX18 containing an internal fragment of *Bcam0227*	This study
pBBR-rpfR	pBBR1MCS containing *rpfR* of H111	(Huber *et al.*, 2002)
pBBR-rpfR$_{AAL}$	pBBR-rpfR harboring an E443A amino acid substitution	This study
pBBR-rpfR$_{GGAAF}$	pBBR-rpfR harboring a D318A and E319A amino acid substitution	This study
pLAFR3-GGDEF-EAL	pLAFR3 containing the encoding region of the GGDEF and EAL domains of RpfR	This study
pLAFR3-GGDEF	pLAFR3 containing the encoding region of the GGDEF domain of RpfR	This study
pLAFR3-EAL	pLAFR3 containing the encoding region of the EAL domain of RpfR	This study
pMLS7-rocR	pMLS7 containing the encoding region of RocR from *P. aeruginosa*	This study
pGEX-rpfR	pGEX-6p-1 containing *rpfR*	This study
pGEX-PAS	pGEX-6p-1 containing the PAS domain of RpfR	This study
pGEX-GGDEF-EAL	pGEX-6p-1 containing the GGDEF and EAL domains of RpfR	This study

Brenner, S. (1974). The Genetics of Caenorhabditis elegans. Genetics 77, 71-94.

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, a J. & Schweizer, H. P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212, 77-86.

Huber, B., Riedel, K., Hentzer, M., Heydorn, a, Gotschlich, a, Givskov, M., Molin, S. & Eberl, L. (2001). The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology 147, 2517-2528.

Huber, B., Riedel, K., Köthe, M., Givskov, M., Molin, S. & Eberl, L. (2002). Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. Molecular microbiology 46, 411-426.

Table S3. PCR primers used in this study

Primer	Sequence (5'-3')
For deletion	
rpfF_{BC}L-F	ggatccgcaccacgtcgaagctctccg
rpfF_{Bc}L-R	aagcttttaggtatgtcctcgtgagatgtggttttaa
rpfF_{Bc}R-F	aagctttaatgcgacgggcgccg
rpfF_{Bc}R-R	tctagagccggtcgagttcatccgtttc
rpfRL-F	ggtacccacatgacgaactcgcgg
rpfRL-R	aagcttggacacgccccgatc
rpfRR-F	aagcttgcgtcgttccggacaagg
rpfRR-R	tctagagccggccgttttacgaag
For in trans *expression*	
rpfF_{Bc}-F	ggatccatgcaactccaatcccatcc
rpfF_{Bc}-R	aagctttacaccgtgcgcagctt
rpfR-F	ggatccatggatgacgaaaacgatagcgc
rpfR-R	aagctttcaggcgatcagcctgagcttt
GGDEF-EAL-F	ggatccatgaacaagttcgtgcagagcggc
GGDEF-EAL-R	aagctttcaggcgatcagcctgagcttt
GGDEF-F	ggatccatgaacaagttcgtgcagagcggc
GGDEF-R	aagctttcactccagcgagaacacgcgatac
EAL-F	ggatccatgaaccagaaggtcgcgaagtaca
EAL-R	aagctttcaggcgatcagcctgagcttt
PA3947--F	ccggaattccggatgaatgatttgaatgttctggtgtt
PA3947-R	tgctctagagcatcaggatccggagcaatagtcg
For protein expression	
rpfR-F'	ggatccatggatgacgaaaacgatagcgc
rpfR-R'	cccgggtcaggcgatcagcctgagcttt
PAS-F	ggatccatggatgacgaaaacgatagcgc
PAS-R	tcccccgggtcagcggaactggaacaggcgc
GGDEF-EAL-F	cgcggatccatgaacaagttcgtgcagagcggc
GGDEF-EAL-R	cccgggtcaggcgatcagcctgagcttt
For plasmid mutagenesis	
AAL-fw	ggcgacgtgcacggcgtcgcggcgctgatccgccagtcg
AAL-rev	cgactggcggatcagcgccgcgacgccgtgcacgtcgcc
GGAAF-fw	gctcgcgcggctcggcggcgccgcattcctcgtgctgttcgaac
GGAAF-rev	gttcgaacagcacgaggaatgcggcgccgccgagccgcgcgagc

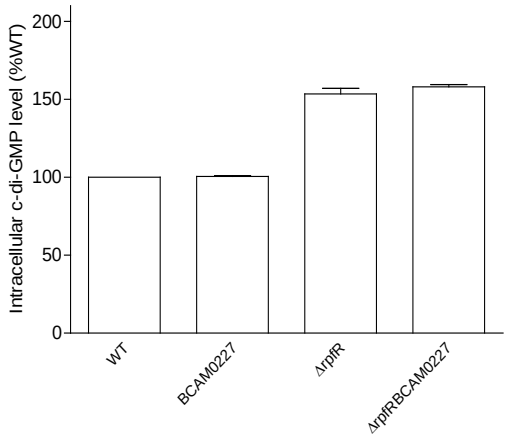
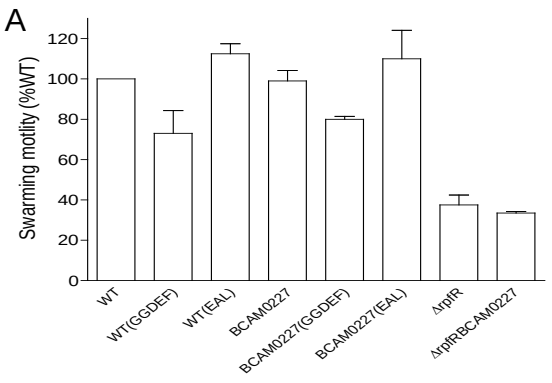


Fig. S1. Influence of BCAM0227 and RpfR on intracellular cyclic-di-GMP level. Detection of intracellular cyclic-di-GMP level by high-performance liquid chromatography (HPLC) assay. The relative amount of cyclic-di-GMP was calculated based on their peak areas. For the convenience of comparison, cyclic-di-GMP level of *B. cenocepacia* wild-type strain H111 was arbitrarily defined as 100% and used to normalize the cyclic-di-GMP level ratios of the other strains. The data shown are the means of two repeats and error bars indicate the standard deviations.



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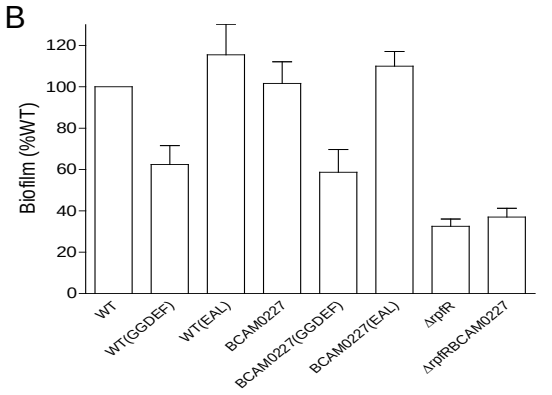

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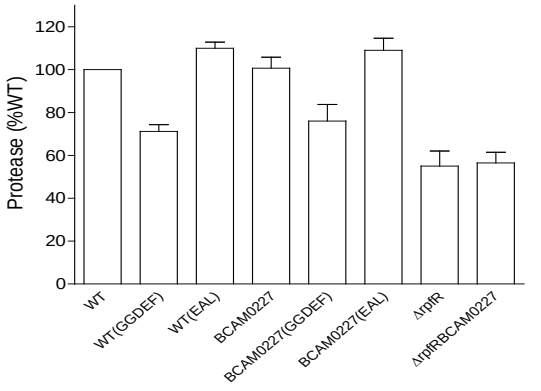


3

4 **Fig. S2**. Mutation of *BCAM0227* does not affect *rpfR*-regulated phenotypes. The wild
5 type, the *BCAM0227* mutant, the rpfRBCAM0227 double mutant, and the derivatives
6 of these strains expressing the coding regions of the GGDEF and EAL domains of
7 RpfR were tested for BDSF-regulated phenotypes. (A) swarming motility, (B), biofilm
8 formation, and (C) protease production. The data shown are the means of three
9 replicates and error bars indicate the standard deviations.

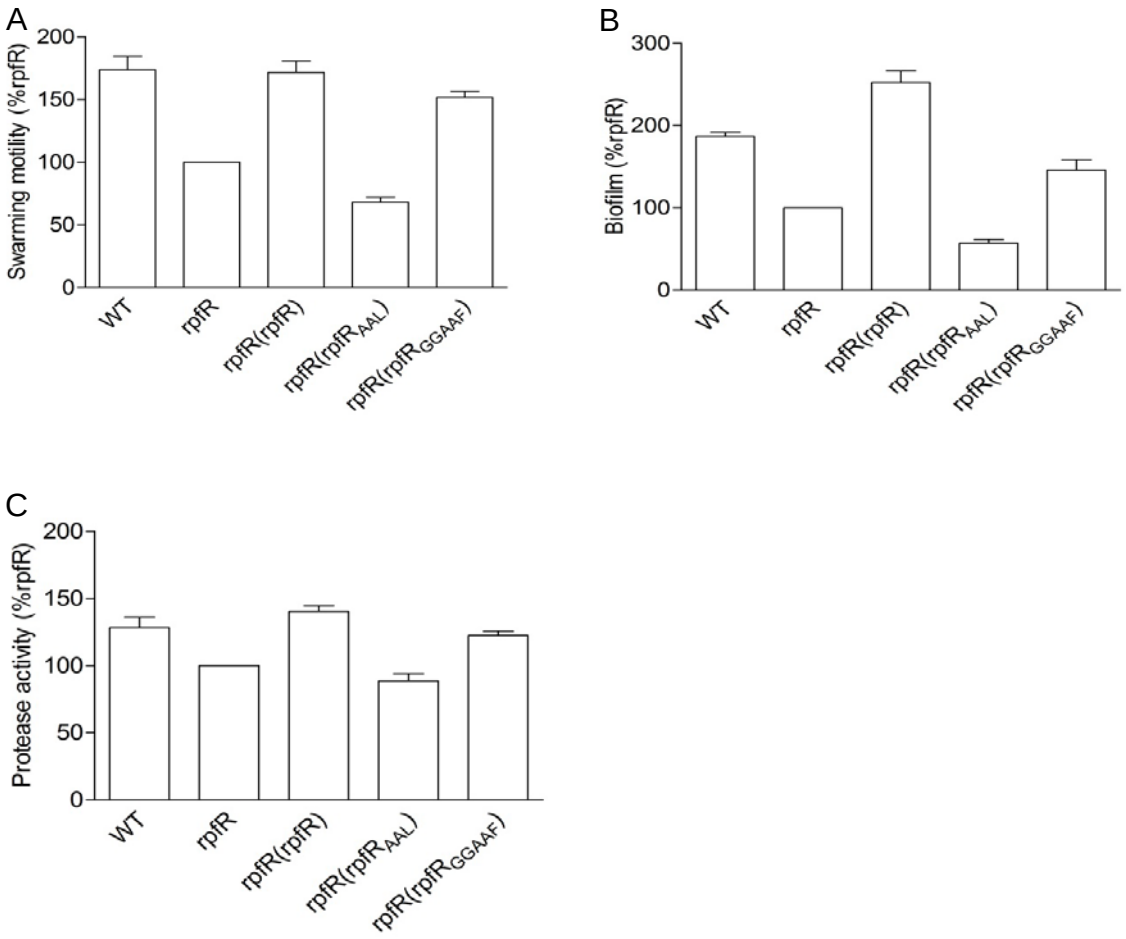


Fig. S3. Complementation of *rpfR* with RpfR, RpfR$_{AAL}$ and RpfR$_{GGAAF}$. *In trans* expression of RpfR and RpfR$_{GGAAF}$ complemented swarming motility (A), biofilm formation (B) and protease activity (C) of a RpfR deficient mutant, whereas *e*xpression of RpfR$_{AAL}$ failed to restore the phenotypic defects of a *rpfR* mutant background. The data shown are the means of three replicates and error bars indicate standard errors.

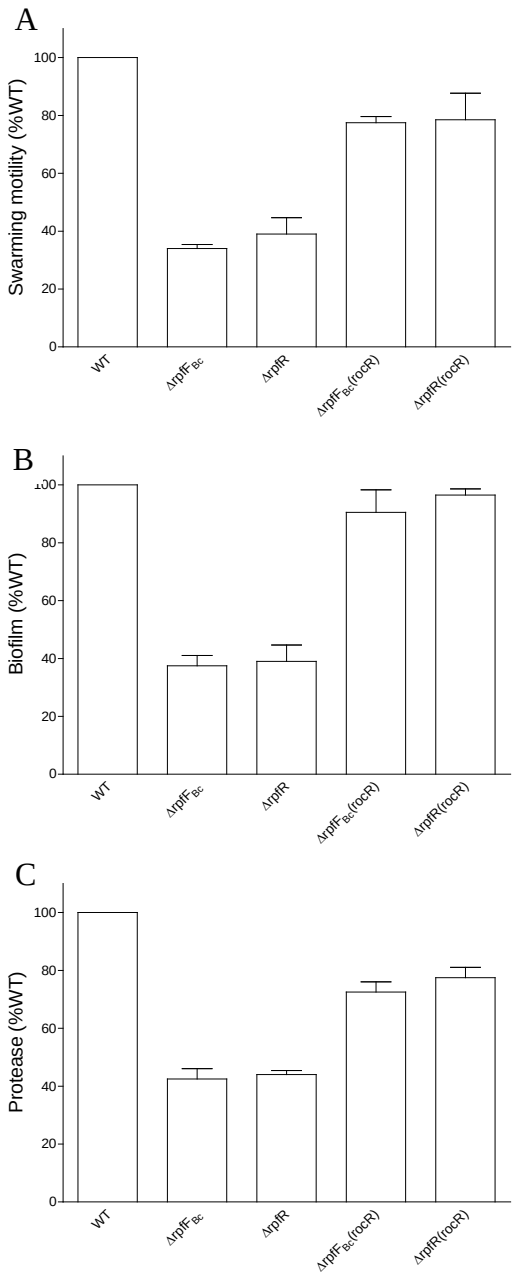


Fig. S4. *In trans* expression of *rocR* (PA3947) from *Pseudomonas aeruginosa* rescued the phenotype defects of ΔrpfF$_{Bc}$ and ΔrpfR in swarming motility (A), biofilm formation (B), and protease production (C). The data shown are the means of two replicates and error bars indicate the standard deviations.

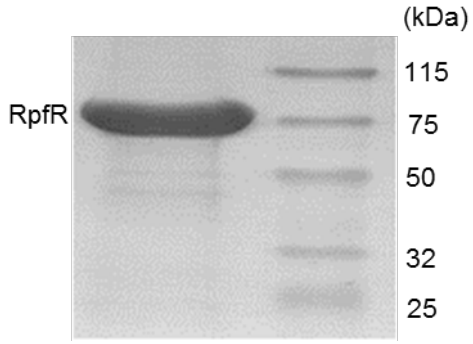


Fig. S5. SDS-PAGE gel electrophoresis of the purified RpfR protein.

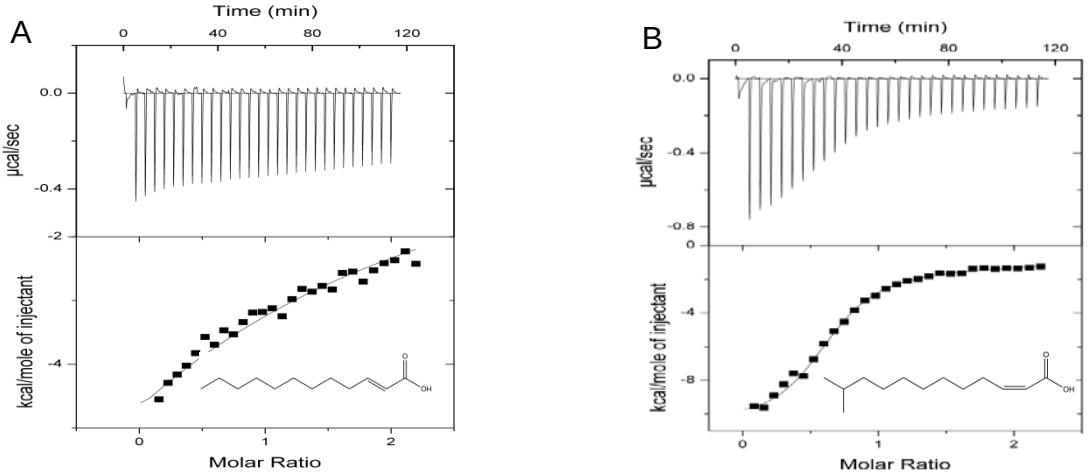
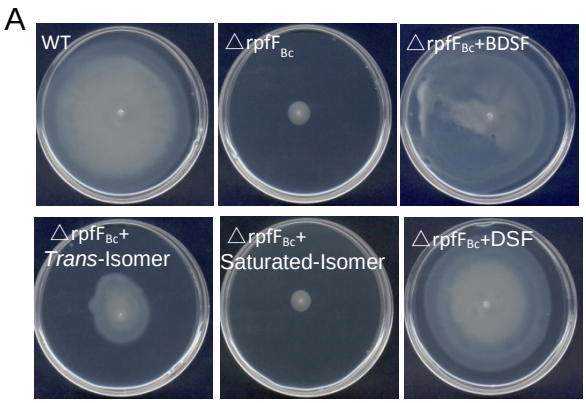
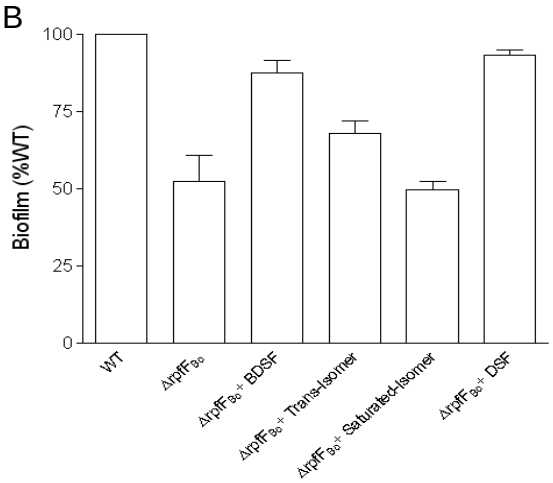


Fig. S6. ITC analysis of interaction between BDSF analogues and RpfR. (A) ITC titration of 20 µM RpfR with 200 µM *trans*-isomer of BDSF in PBS buffer at 21°C. (B) ITC titration of 20 µM RpfR with 200 µM DSF in PBS buffer at 21°C.





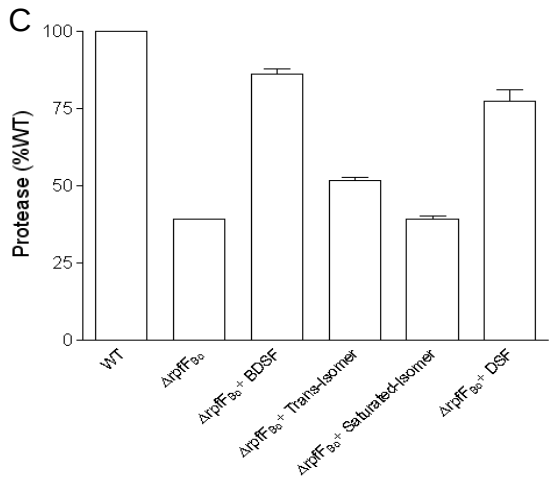


Fig. S7. Effect of BDSF and analogues on BDSF-regulated phenotypes. (A) Swarming motility, (B) biofilm formation, and (C) protease production. The compounds were added separately at a final concentration of 5 μM. The data shown are the means of three replicates and error bars indicate the standard deviations.

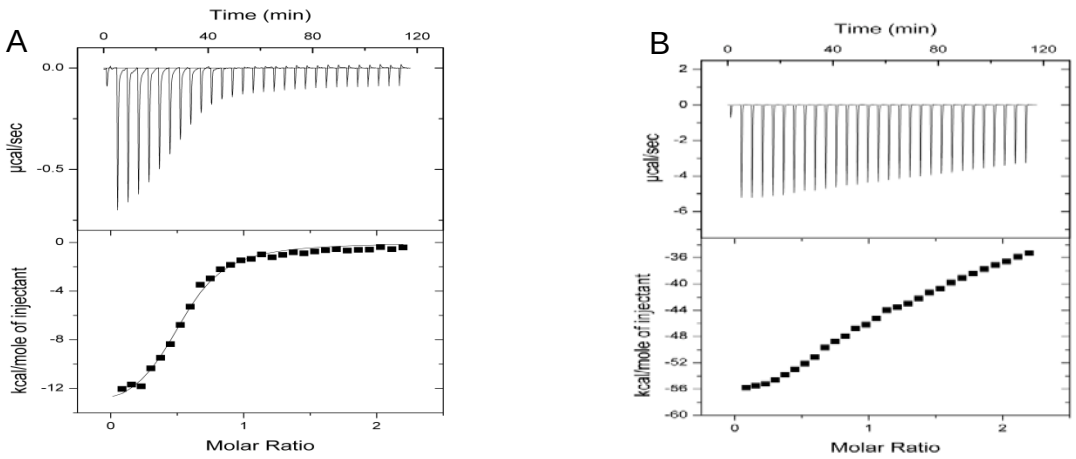


Fig. S8. ITC analysis of interaction between BDSF and RpfR domains. (A) ITC titration of 20 µM PAS domain of RpfR with 200 µM BDSF in PBS buffer at 21°C. (B) ITC titration of 20 µM GGDEF-EAL domain of RpfR with 200 µM BDSF in PBS buffer at 21°C.

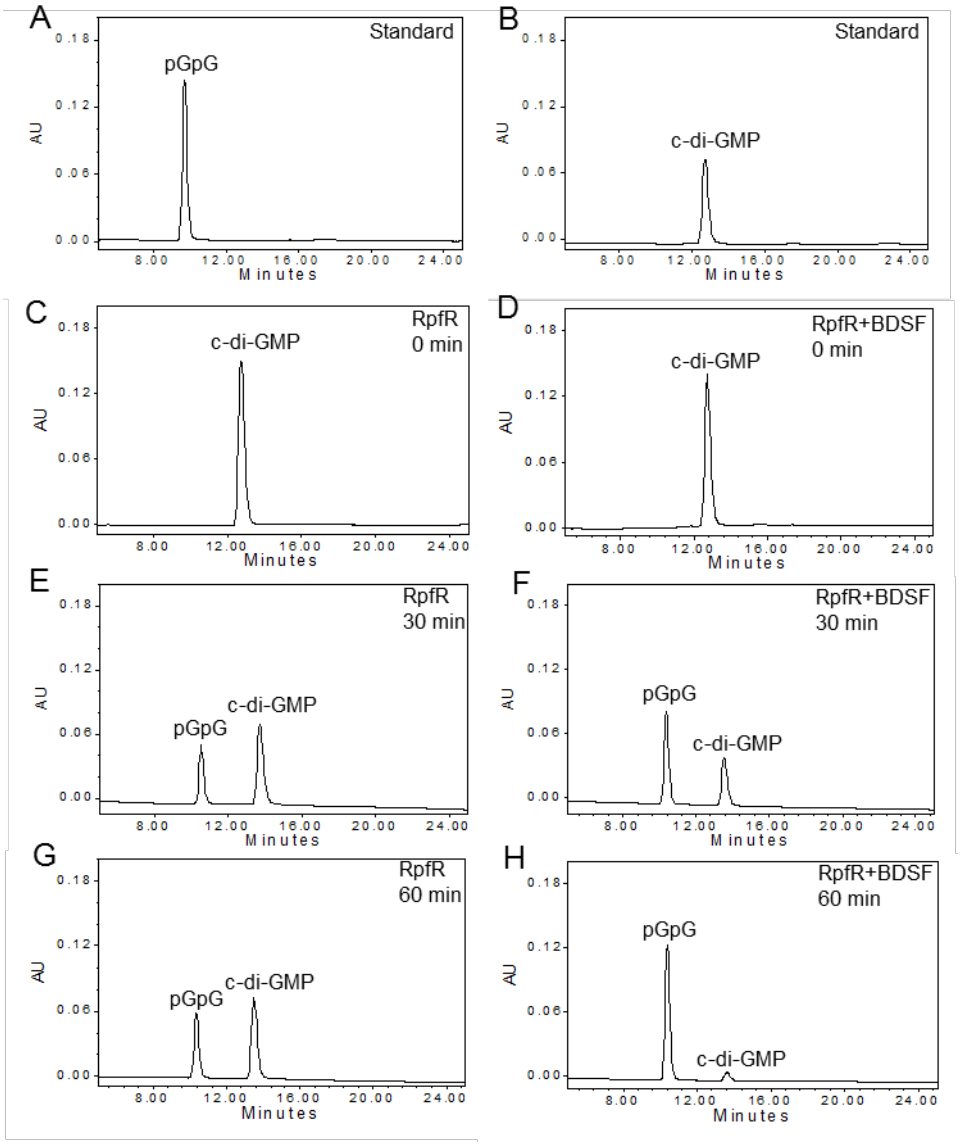


Fig. S9. HPLC analysis of RpfR cyclic-di-GMP phosphodiesterase activity. HPLC analysis of the standard pGpG (A) and cyclic-di-GMP (B). The HPLC chromatograms in the absence and presence of BDSF at 0 min (C, D), 30 min (E, F), and 60 min (G, H), respectively.

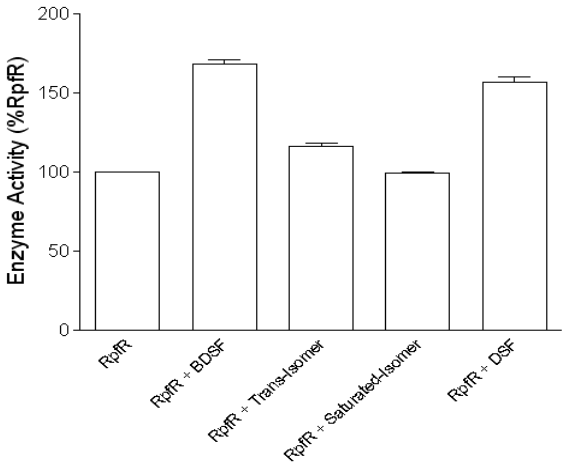


Fig. S10. Effects of BDSF and analogues on RpfR enzyme activity. For the convenience of comparison, enzyme activity of RpfR at 30 min was defined as 100% and used to normalize the cyclic-di-GMP degradation activity of RpfR in the presence of different ligands. The data shown are the means of two replicates and error bars indicate the standard deviations.

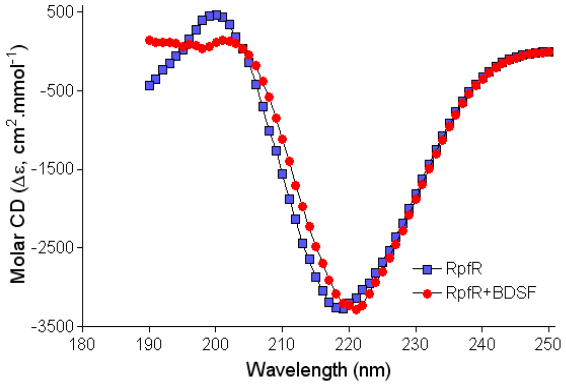


Fig. S11. Impact of BDSF on RpfR protein conformation.