

Supplementary information

The Wsp chemosensory system modulates c-di-GMP-dependent biofilm formation by integrating DSF quorum sensing through the WspR-RpfG complex in *Lysobacter*

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Content: 2 Supplementary Tables, 13 Supplementary Figures.

Supplementary Table 1. Strains and plasmids used in this study

Strains and plasmids	Characteristics ^a	Source
Wild-type strains		
<i>Lysobacter enzymogenes</i> OH11	Wild type, Km ^R	1
In-frame deletion mutants		
$\Delta wspA$	In-frame deletion of <i>wspA</i> in strain OH11, Km ^R	2
$\Delta wspB$	In-frame deletion of <i>wspB</i> in strain OH11, Km ^R	2
$\Delta wspC$	In-frame deletion of <i>wspC</i> in strain OH11, Km ^R	2
$\Delta wspD$	In-frame deletion of <i>wspD</i> in strain OH11, Km ^R	2
$\Delta wspE$	In-frame deletion of <i>wspE</i> in strain OH11, Km ^R	2
$\Delta wspF$	In-frame deletion of <i>wspF</i> in strain OH11, Km ^R	3
$\Delta wspR$	In-frame deletion of <i>wspR</i> in strain OH11, Km ^R	2
$\Delta lchP$	In-frame deletion of <i>lchP</i> in strain OH11, Km ^R	4
$\Delta rpfG$	In-frame deletion of <i>rpfG</i> in strain OH11, Km ^R	4
$\Delta wspF$ - <i>wspF</i> ^{D53A}	Chromosomal mutation of D53→A53 of WspF in wild-type, Km ^R	This study
$\Delta wspF$ - <i>wspF</i> ^{D53E}	Chromosomal mutation of D53→E53 of WspF in wild-type, Km ^R	This study
$\Delta wspF$ - <i>wspF</i> ^{S115A}	Chromosomal mutation of S115→A115 of WspF in wild-type, Km ^R	2
$\Delta wspF$ - <i>wspF</i> ^{H182A}	Chromosomal mutation of H182→A182 of WspF in wild type, Km ^R	2
$\Delta wspF\Delta wspR$ - <i>wspR</i> ^{D72A}	In-frame deletion of <i>wspF</i> and chromosomal D72A mutation in the <i>wspR</i> gene, Km ^R	2
$\Delta wspF\Delta wspR$ - <i>wspR</i> ^{GGAAF}	Chromosomal mutation of GGEEF→GGAAF of WspR in the <i>wspF</i> mutation, Km ^R	2
$\Delta wspA\Delta rpfF$	Both in-frame deletion of <i>wspA</i> and <i>rpfF</i> in strain OH11, Km ^R	This study
$\Delta wspR\Delta rpfF$	Both in-frame deletion of <i>wspR</i> and <i>rpfF</i> in strain OH11, Km ^R	This study
$\Delta wspA\Delta wspF$	Both in-frame deletion of <i>wspA</i> and <i>wspF</i> in strain OH11, Km ^R	2
$\Delta wspE\Delta wspF$	Both in-frame deletion of <i>wspE</i> and <i>wspF</i> in strain OH11, Km ^R	2
$\Delta wspF\Delta wspR$	Both in-frame deletion of <i>wspF</i> and <i>wspR</i> in strain OH11, Km ^R	2
$\Delta wspF\Delta 0901$	Both in-frame deletion of <i>wspF</i> and <i>0901</i> in strain OH11, Km ^R	2
$\Delta wspF\Delta lchD$	Both in-frame deletion of <i>wspF</i> and <i>3756</i> in strain OH11, Km ^R	2

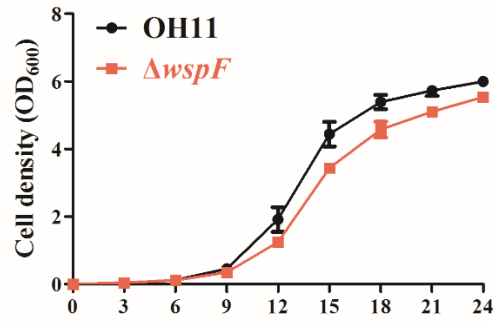
Complementary and overexpression strains		
$\Delta wspF$ (pBBR)	$\Delta wspF$ harbouring plasmid pBBR, Km ^R , Gm ^R	This study
$\Delta wspF(yhjH)$	$\Delta wspF$ harbouring plasmid pBBR- <i>yhjH</i> , Km ^R , Gm ^R	This study
$\Delta wspF$ (GFP)	$\Delta wspF$ harbouring plasmid pBBR-GFP, Km ^R , Gm ^R	This study
$\Delta rpfG$ (GFP)	$\Delta rpfG$ harbouring plasmid pBBR-GFP, Km ^R , Gm ^R	This study
$\Delta rpfG(rpfG)$	$\Delta rpfG$ harbouring plasmid pBBR- <i>rpfG</i> , Km ^R , Gm ^R	5
$\Delta rpfG(rpfG^{H190A})$	$\Delta rpfG$ harbouring plasmid pBBR- <i>rpfG</i> ^{H190A} , Km ^R , Gm ^R	5
$\Delta rpfG(rpfG^{Y254A})$	$\Delta rpfG$ harbouring plasmid pBBR- <i>rpfG</i> ^{Y254A} , Km ^R , Gm ^R	5
OH11(<i>wspR</i>)	OH11 harbouring plasmid pBBR- <i>wspR</i> , Km ^R , Gm ^R	2
OH11(<i>wspR</i> ^{GGAFF})	OH11 harbouring plasmid pBBR- <i>wspR</i> ^{GGEFF⁻GGAFF} , Km ^R , Gm ^R	2
OH11(<i>wspC</i>)	OH11 harbouring plasmid pBBR- <i>wspC</i> , Km ^R , Gm ^R	2
OH11(<i>lchD</i>)	OH11 harbouring plasmid pBBR- <i>lchD</i> , Km ^R , Gm ^R	This study
<i>Escherichia coli</i>		
DH5 α	Host strain for molecular cloning	6
XL1-Blue MRF ⁺ Kan	Host strain for bacterial two-hybrid assay	7
BL21(DE3)	Host strain for protein expression	8
Plasmids		
pEX18GM	Suicide vector with a <i>sacB</i> gene, Gm ^R	9
pBBR1-MCS5	Broad-host- vector with a P _{lac} promoter, Gm ^R	10
pTRG	The plasmid used for protein expression in bacterial two-hybridization assay, Tet ^R	7
pBT	The plasmid used for protein expression in bacterial two-hybridization assay, Chlo ^R	11
pET30a	Protein expression vector with a His tag, Km ^R	8
pEX18-WspA	pEX18GM with two flanking fragments of <i>wspA</i> , Gm ^R	2
pEX18-WspR	pEX18GM with two flanking fragments of <i>wspR</i> , Gm ^R	2
pEX18- <i>wspF</i> ^{D53A}	pEX18GM with fragment containing coding region of <i>wspF</i> ^{D53A} and its two flanking fragments of <i>wspF</i> , Gm ^R	This study
pEX18- <i>wspF</i> ^{D53E}	pEX18GM with fragment containing coding region of <i>wspF</i> ^{D53E} and its two flanking fragments of <i>wspF</i> , Gm ^R	This study
pBBR- <i>yhjH</i>	pBBR1-MCS5 with fragment <i>yhjH</i> , Gm ^R	4
pET30a-WspR	pET30a with the coding region of <i>wspR</i> , Km ^R	2
pET30a-WspR ^{GGEFF⁻GGAFF}	pET30a with the coding region of <i>wspR</i> ^{GGAFF} , Km ^R	2
pET30a-WspR ^{AREC}	pET30a with the coding region of <i>wspR</i> ^{AREC} , Km ^R	2
pET30a-RpfG-FLAG	pET30a containing the coding region of <i>rpfG</i> with C-FLAG	This study
pET30a-HD-GYP-FLAG	pET30a containing the coding region of HD-GYP domain of <i>rpfG</i> with C-FLAG	This study
pMAL-p2x	Protein expression vector with a MBP tag, Amp ^R	5
pMAL-RpfG	pMAL with the coding region of <i>rpfG</i> , Amp ^R	5
pMAL-RpfG ^{H190A}	pMAL with the coding region of <i>rpfG</i> ^{H190A} , Amp ^R	5
pTRG-WspR	pTRG with the coding region of <i>wspR</i> from strain OH11, Tet ^R	2
pBT-RpfG	pBT with the coding region of <i>rpfG</i> from strain OH11, Chl ^R	This study

^aKm^R, Gm^R Amp^R, Tet^R, Chlo^R, kanamycin, gentamicin, ampicillin, tetracycline, chloramphenicol resistance, respectively.

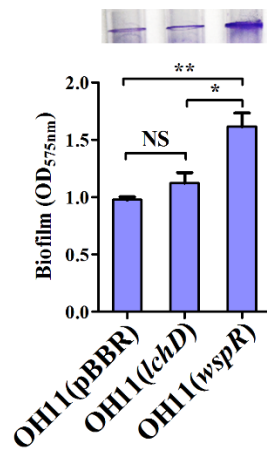
Supplementary Table 2. Primers used in this study

Primer	Sequence (5'-3') ^a	Use
Point mutation		
<i>wspF</i> ^{D53A} -F1	GGGGTACCTGGGCAAGGTCAAGGACATC(<i>KpnI</i>)	To amplify a 760-bp upstream of <i>wspF</i> ^{D53A}
<i>wspF</i> ^{D53A} -R1	CGTTCATGCCGGGCATGACCAGGGCCATCAGCACCA GGTCGG	
<i>wspF</i> ^{D53A} -F2	CCGACCTGGTGCTGATGGCCCTGGTCATGCCCGGCAT GAACG	To amplify a 1362-bp downstream of <i>wspF</i> ^{D53A}
<i>wspF</i> ^{D53A} -R2	CCCAAGCTTATCAGGCGTTTCTGGTGCTC(<i>HindIII</i>)	
Protein expression		
<i>rpfG</i> -FLAG-F	GGAATTCCATATGCTGCGCCACATCATCGA GGACATTG (<i>NdeI</i>)	To amplify a 1041-bp fragment containing coding region of <i>rpfG</i> fused FLAG tag
<i>rpfG</i> -FLAG-R	CCCAAGCTTTTACTTGTCATCGTCGTCCTTGT AATCCTCCAGGCCGGGCCTGCTGGGTAC (<i>HindIII</i>)	
HD-GYP-FLAG-F	GGAATTCCATATGCGCGACGCCGGCACCAGC GCCTACCTGGAGCGC (<i>NdeI</i>)	To amplify a 414-bp fragment containing coding region of HD-GYP domain of <i>rpfG</i> fused FLAG tag
HD-GYP-FLAG-R	CCCAAGCTTTTACTTGTCATCGTCGTCCTTGT AATCGGCCTTCTTGTACGGACGCGGCG (<i>HindIII</i>)	
bacterial two hybrid		
pBT- <i>rpfG</i> -F	GAAGAGACGTTTGGCGCGGCCGCAAT GCTGCGCCACATCATCGAGGACATTG	To amplify a 1017-bp fragment containing coding region of <i>rpfG</i>
pBT- <i>rpfG</i> -R	TTAACTCGAGGATCCCCGGAATTCTTA CTCCAGGCCGGGCCTGCTGGGTAC	

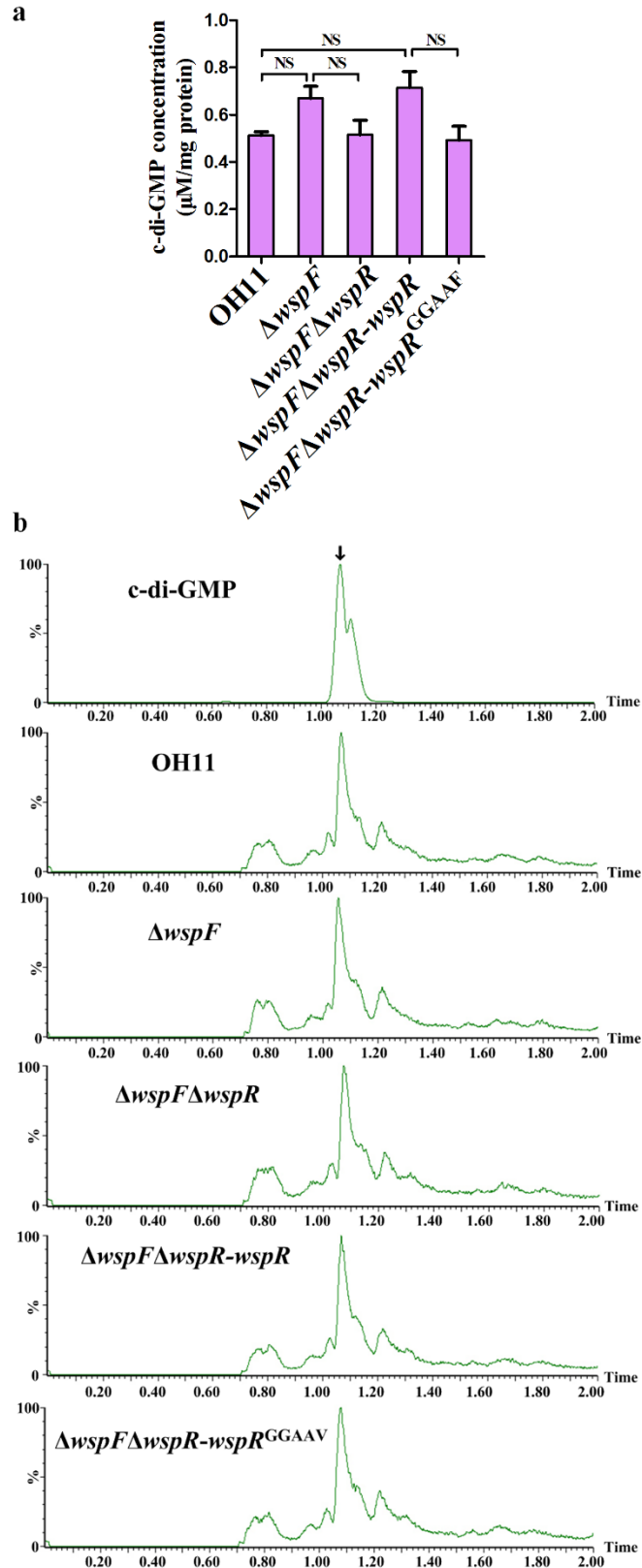
^a Restricted digestion enzyme site was underlined.



Supplementary Figure 1. Effects of WspF on the growth ability of *L. enzymogenes* OH11 in LB broth.

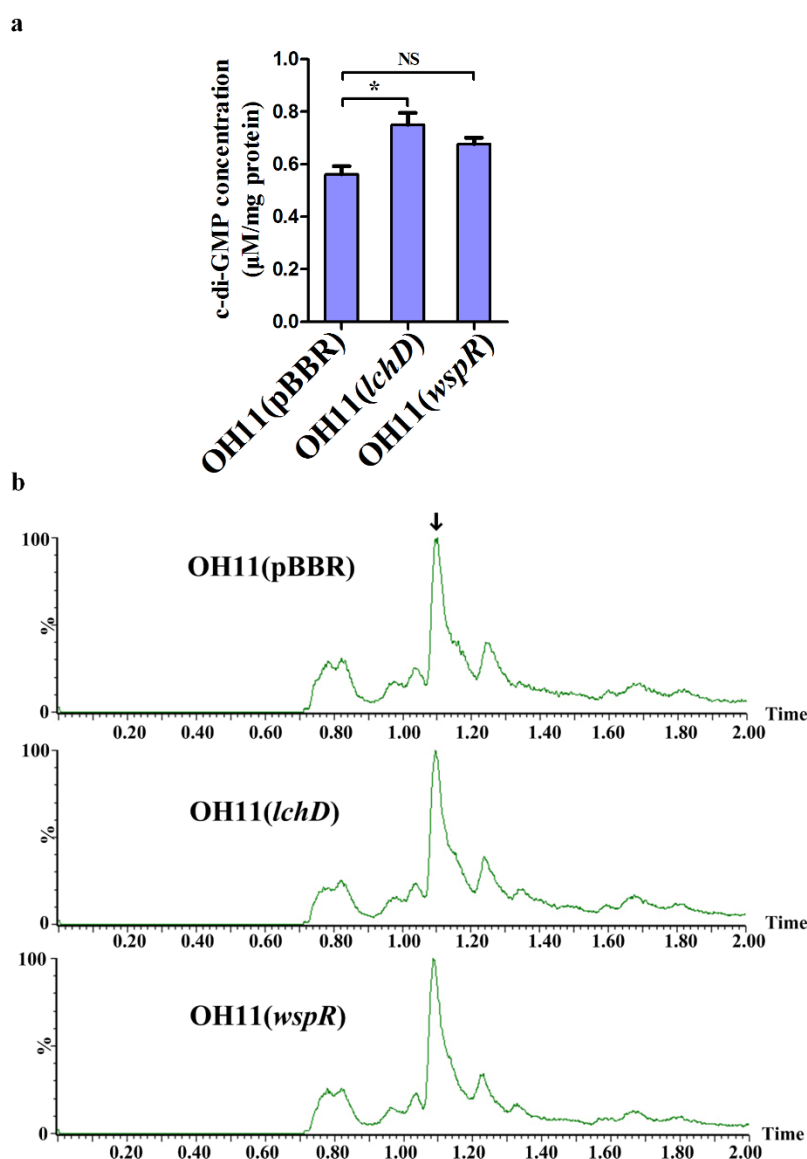


Supplementary Figure 2. Biofilm formation in *wspR* and *lchD* overexpressing strains. OH11, wild-type strain. pBBR, short for pBBR1-MCS5, is a vector control.

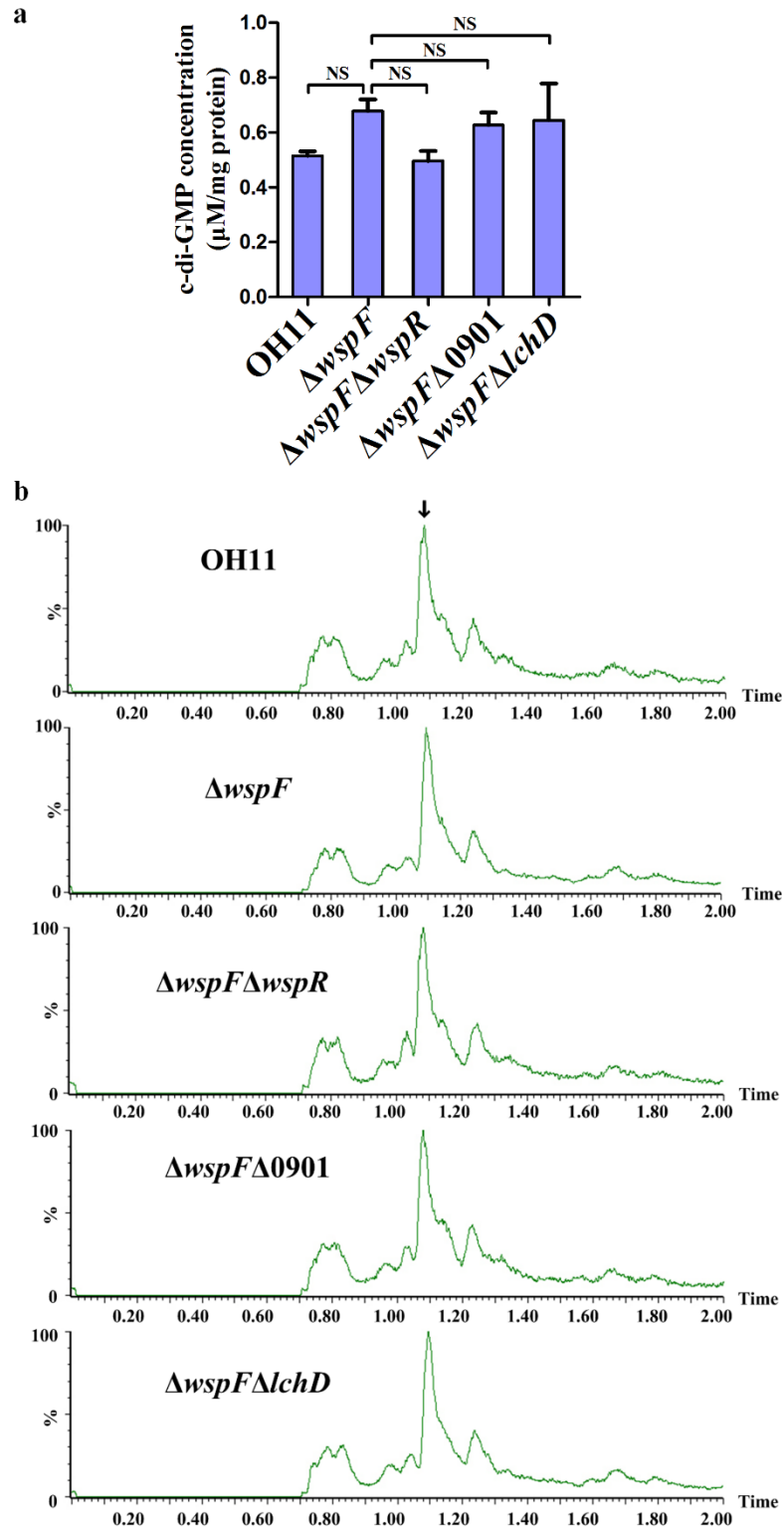


Supplementary Figure 3. Total intracellular c-di-GMP concentration determined by LC-MS/MS. a The total c-di-GMP level of all tested strains remained consistent with the wild type. **b** LC-MS/MS chromatogram corresponding to **a**. The black arrow represents the peak time of c-di-GMP. Statistical comparisons were performed using

one-way ANOVA of GraphPad software (GraphPad, La Jolla, CA). In panel **a**, the mean data of $\pm$ SD from three experiments were shown, $**P < 0.01$. NS means not significant.

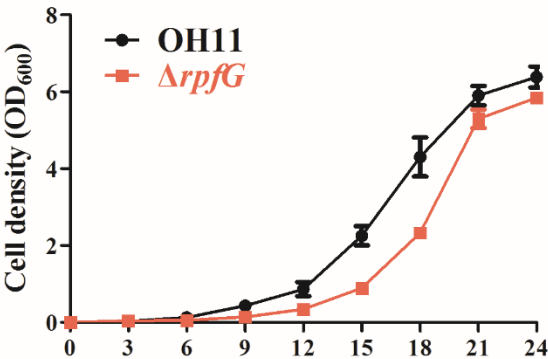


Supplementary Figure 4. Total c-di-GMP level of *lchD* and *wspR* overexpressing strains determined by LC-MS/MS. **a** The total c-di-GMP level of *lchD* overexpressing strains was modestly higher than that of the wild type, while the *wspR* overexpressing strains did not change the total content of c-di-GMP. **b** LC-MS/MS chromatogram corresponding to **a**. The black arrow represents the peak time of c-di-GMP. Statistical comparisons were performed with using one-way ANOVA of GraphPad software (GraphPad, La Jolla, CA). In panel **a**, mean data $\pm$ SD from three experiments were shown, $*P < 0.05$. NS means not significant.

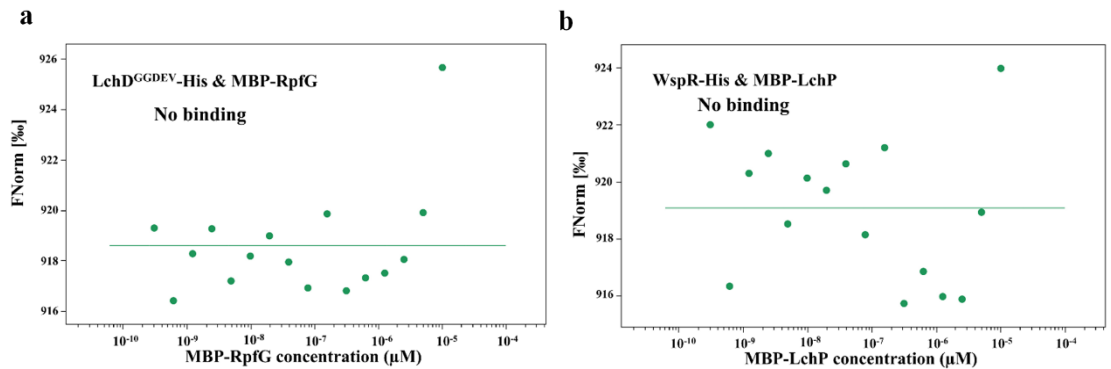


Supplementary Figure 5. Total c-di-GMP level of *wspF0901* and *wspF Δ lchD* double mutants determined by LC-MS/MS. a The total c-di-GMP level of all tested strains remained consistent with the wild type. **b** LC-MS/MS chromatogram corresponding to **a**. The black arrow represents the peak time of c-di-GMP. Statistical comparisons were performed using one-way ANOVA of GraphPad software (GraphPad, La Jolla, CA). In

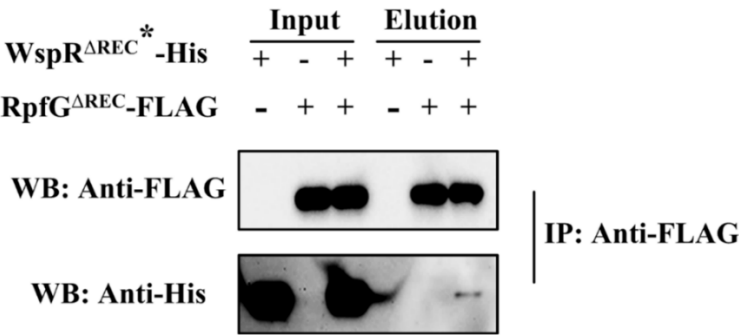
panel **a**, mean data $\pm$ SD from three experiments were shown, $**P < 0.01$. NS means not significant.



Supplementary Figure 6. Effects of RpfG on the growth ability of *L. enzymogenes* OH11 in LB broth.

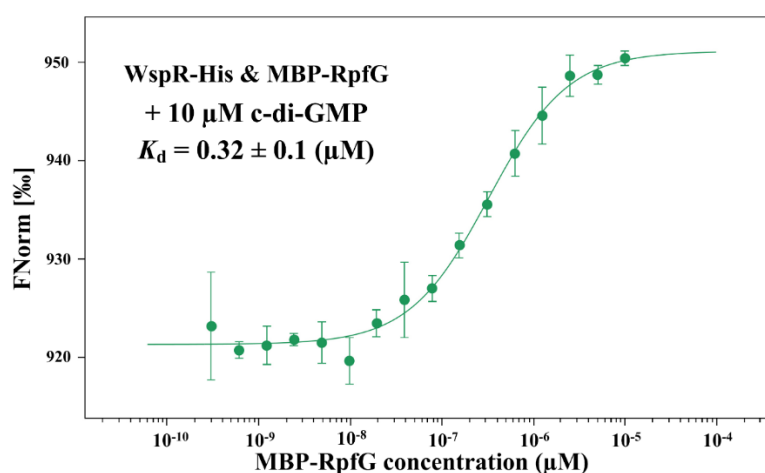


Supplementary Figure 7. MST analysis showed that the intracellular portion of LchD (LchD^{GGDEV}-His) did not interact with RpfG (a), and WspR did not interact with MBP-LchP (b).

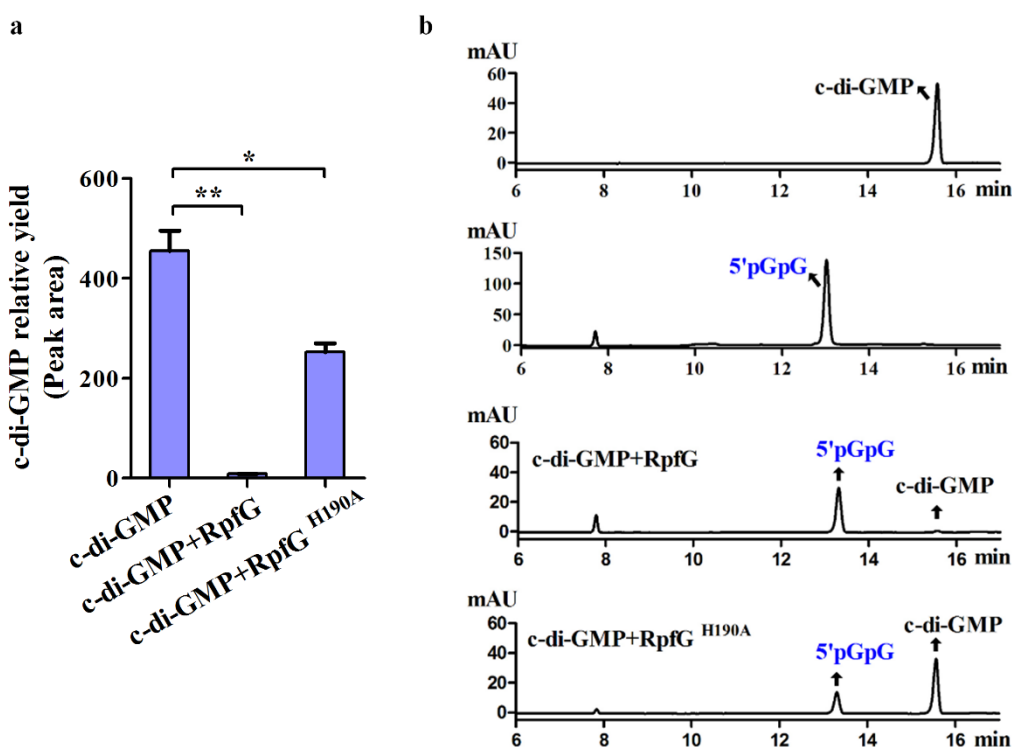


Supplementary Figure 8. The HD-GYP domain of RpfG interacts with the enzymatically inactive GGDEF domain of WspR. A pull-down assay confirming the direct interaction of the HD-GYP domain of RpfG (RpfG^{ΔREC}) with the enzymatically

inactive GGDEF domain of WspR (WspR^{ΔREC*}) by changing the “GGDEF” motif to “GGAAF”. IP assay was performed using anti-FLAG antibody. Western blotting was performed using anti-FLAG and anti-His antibodies. All blots derive from the same experiment and that they were processed in parallel.

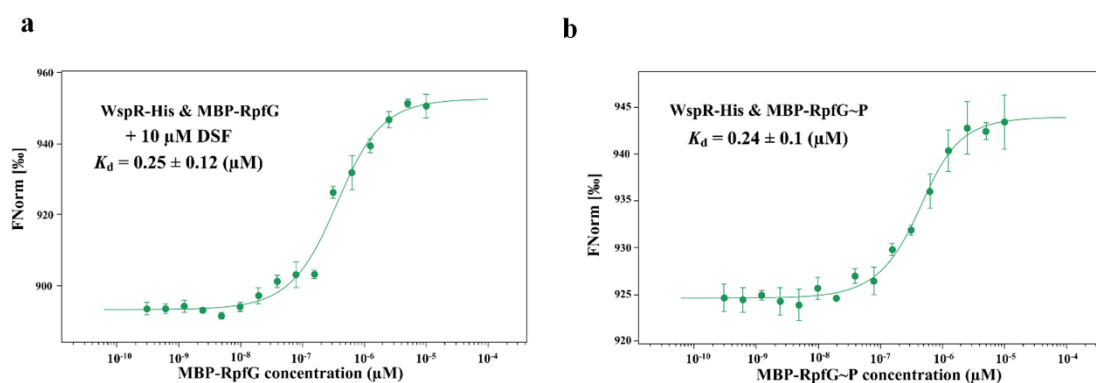


Supplementary Figure 9. The physiological range of c-di-GMP does not affect WspR-RpfG binding. MST showed that WspR-His still interacted with MBP-RpfG with moderate affinity (K_d , 0.32 μ M) in the presence of 10- μ M c-di-GMP.

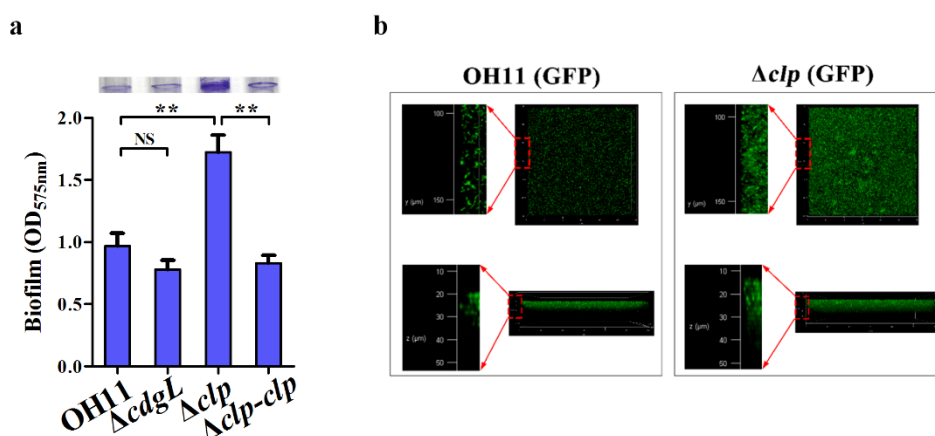


Supplementary Figure 10. RpfG is active in degrading c-di-GMP *in vitro* as

detected by HPLC. **a** Purified MBP-RpfG is highly active while MBP-RpfG^{H190A} is moderately active against c-di-GMP *in vitro*. **b** HPLC chromatogram corresponding to **a**. The c-di-GMP and 5'pGpG standard are shown in black and blue, respectively. Statistical comparisons were performed using one-way ANOVA of GraphPad software (GraphPad, La Jolla, CA). In panels **a**, mean data $\pm$ SD from three experiments were shown, $**P < 0.01$.

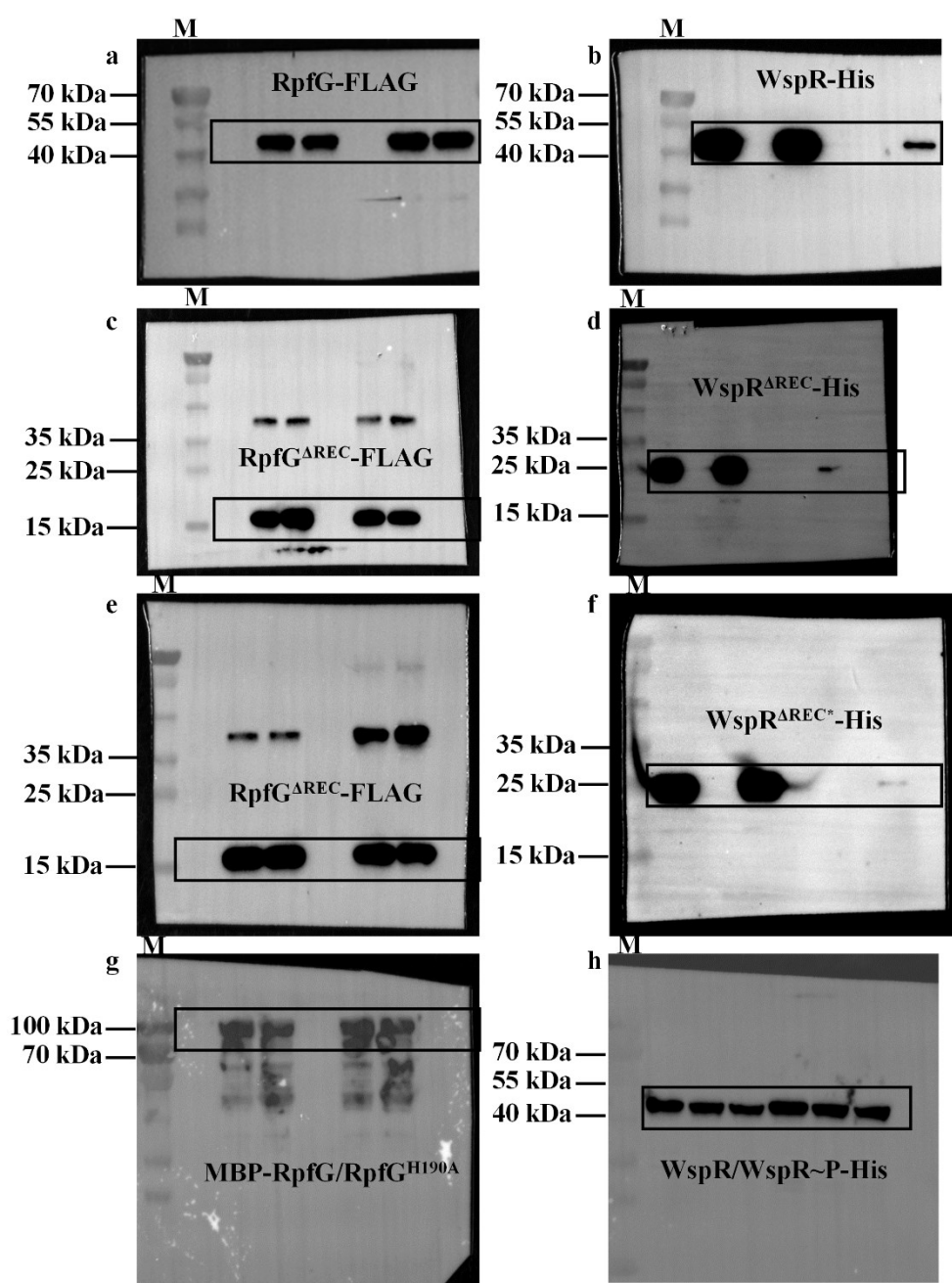


Supplementary Figure 11. DSF or RpfG phosphorylation did not affect WspR-RpfG binding. **a** MST showed that WspR-His still interacted with MBP-RpfG with moderate affinity (K_d , 0.25 μ M) in the presence of 10- μ M DSF. **b** MST showed that RpfG phosphorylation still interacted with WspR-His with moderate affinity (K_d , 0.24 μ M).



Supplementary Figure 12. Clp, a well-known c-di-GMP-binding transcription factor, negatively regulated biofilm formation in *L. enzymogenes*. **a** Clp, a c-di-GMP receptor, but not CdgL, positively regulated biofilm formation. Δ clp-clp, the native *clp*, was chromosomally inserted into the *clp* mutant. **b** CLSM of biofilms formed by wild-

type and *clp* mutants. The top faces (y and x axis) of the three-dimensional biofilm structure formed by the tested strains was shown in the upper part of the black box. The side faces (z and x axis) of the three-dimensional biofilm structures formed by the test strains were shown in the lower part of the black box. In each section, an enlarged view of the red circle was presented on the left. Statistical comparisons were performed using one-way ANOVA of GraphPad software (GraphPad, La Jolla, CA). In panels **a**, mean data $\pm$ SD from three experiments were shown, $**P < 0.01$. NS means not significant.



Supplementary Figure 13. Uncropped gels & blots. a&b Box highlighted by black

corresponds to Fig. 4b. **c&d** Box highlighted by black corresponds to Fig. 4f. **e&f** Box highlighted by black corresponds to Supplementary Fig. 8. **g&h** Box highlighted by black corresponds to Fig. 5f & 6e.

Supplementary References

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