Vibrio parahaemolyticus RhsP Represents a Widespread Group of Pro-effectors for Type VI Secretion System

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Running title: A widespread pro-effector for T6SS

Keywords: T6SS, Rhs, Pro-effector, PAAR, social cheater

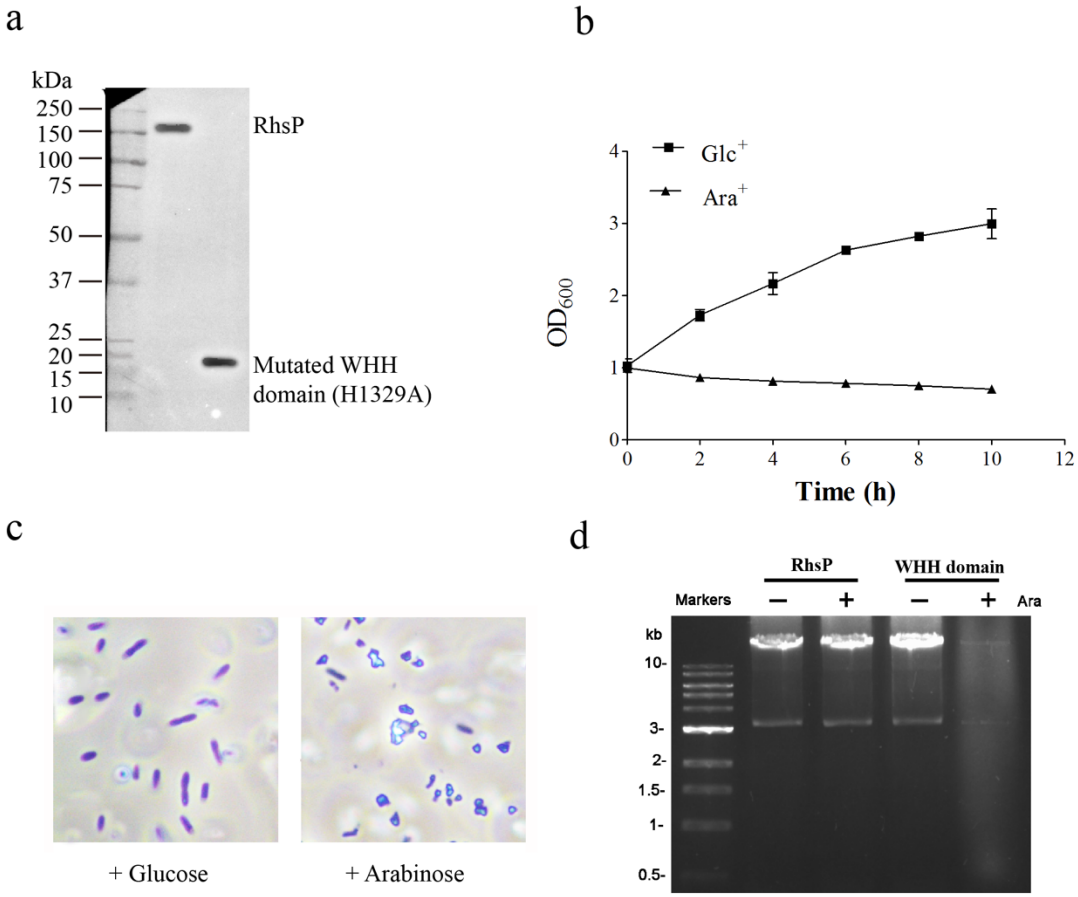
Supporting Information

Supplementary Methods

Quantitative Real-time PCR. The total bacterial RNA was extracted using RNeasy Mini Kit (Qiagen) after being treated with RNAprotect Bacteria Reagent (Qiagen). Synthesis of cDNA was performed using PrimeScipt RT reagent Kit (Takara). Quantitative PCR was carried on cDNA using SYBR Premix Ex TagII (Takara) on a CFX96 Real-Time PCR machine (BioRad). 16S rRNA gene was used as an internal control.

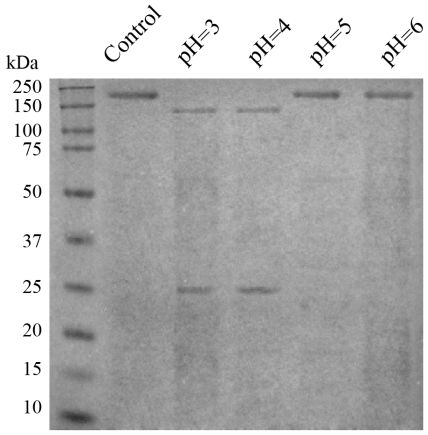
Microscopy imaging of bacteria. *E. coli* cells containing pBAD24-WHH domain were recovered in 5ml LB to OD_{600} = 0.6, and induced by 0.2% arabinose and glucose respectively for 2 hours. Five microliters of bacteria were dropped on the object slide and imaged using a Nikon eclipse E200 microscope.

Supplementary Figures



Supplementary Fig. 1. Toxicity of RhsP and its WHH domain on *E. coli*. (a) Western blot analysis of the expression of RhsP-FLAG in *E. coli* DH5α. A WHH domain with site-directed mutagenesis (H1329A) expressed in *E. coli* DH5α was included as a control. (b) Growth curve of *E. coli* expressing WHH domain (pBAD24-WHH domain-F). 0.2 % glucose (Glc^+) or 0.2% arabinose (Ara^+) was added into cultures when the bacterial OD_{600} reached 1.0 to repress or induce the expression of WHH domain respectively. Bacterial number as measured by OD_{600} decreased gradually when the expression of WHH domain was induced by arabinose, contrary to the increase when bacteria were supplemented with glucose. (c) Microscopy images of *E. coli* expressing WHH domain (pBAD24-WHH domain-F). Cells died and broke upon WHH domain induction, while remained intact when the expression of WHH domain was repressed. Images were captured under 100 × visual field. (d) The electrophoresis of the DNA extracted from *E. coli* containing pBAD24-*rhsP-F* or pBAD24-WHH domain-F. The expression of RhsP and WHH domain in *E. coli* DH5α was induced by arabinose for 4 hours and the total DNA from bacterial cells was extracted and analysed by 1% agarose gel.

a



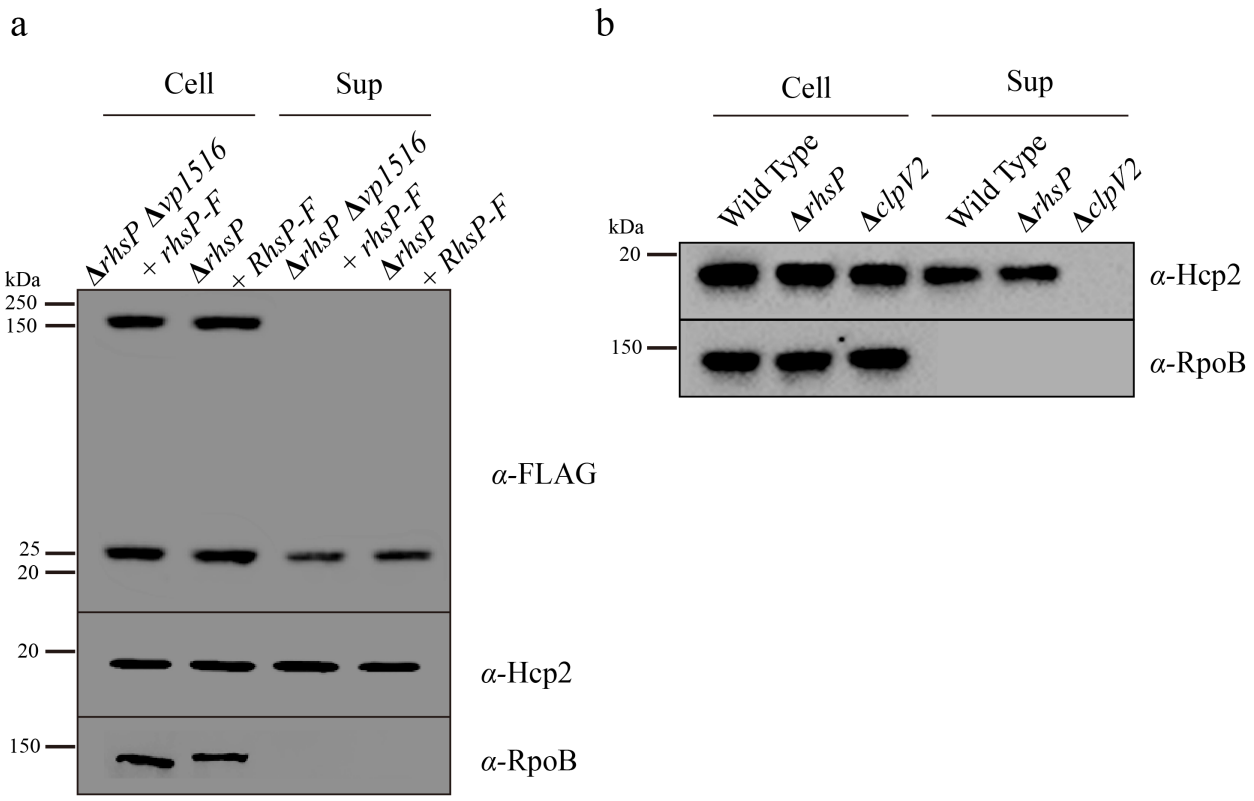
b

MIPQFVIPLTNCLGQSYHFSSQPIPKGEHKKFDSEQSAKAFLDDFVPLRSSRVEELYHLLGQ
FPPNVPDEELTPELYAPVFAKALVNGSLYVASFPKTKKNATISSEPTPVPKQVKAKSKQNK
AHTSSKTQAKNSASAKPLQTGSECHEKAGDPVSLVTGEEILTLNDVELPNGFVWSRTYRS
SKASRNQGLGYGWRHAFQFELKEVTDEKHNVTSWEFISDSADEIEFEPVEHGSTSYQVYV
GASCHFLNPNIRIVTLSSGDQYRFELVEDIWLLKQVRNGIFSTFQLRYSRNHRLIEVAHNK
RPVLECQYDKQGRLVELLNAKTEQVLTTYIYDEQDDLVGATNDLGLTERYEYQDQHLIA
KRVRPTGFTHYFEWSGEGSSAKCIRNFGDSGIYDYRFHYEGAKSSYSDSLDNEWTFIHDE
QGHLLEKSSPTGRTWQWHYDHLGRKEKAVFPDNSTTQYQYNQQGQLISKLHSSGAQIQY
GYDSLGKLVKTVSPDGDLEKAYYNSLGQRVWDIDALGCVTEYEYDKHGQVVKRESEDG
KKSRWWWDKQQRLVAHEVDGTLLRYSYGATDLVNGIAYPDGCVAQISYDDYGRRTSIR
YFNDEDKVGYSEEYAYDEFSRVAQIQTPEGVTSYQWGALAQQEAVIFPDGSHISYEYDQ
QRNLTKLVRSDGLAFEFWYDSEGLLSGTVGFDGLHSQFKYDSMGRIIRKDVADRTVLYS
YDDAGFLQHIKAGNGKNIVENHFNYTLGGRLTLASNRHQTLQYQYSSFGHLTKRIQGQFE
IGEEFNRVGQRVSQTLPDKTSFNFSYDTNGRLSEIRFSDDSLPKIEFQYDVMGRLSVTETES
FRESKLYDGVGRLVEQQWSGREKKYIYNAQNRISSILDNTAGATHYQYDTLGYVTKVSE
AGSTSTFESDSFGNPALADSKVMSDRIEAYAGVRYKYDQQGNQVKREGDGTVQKRVFD
ALSQLVEVHGDSSISHYEYDALGRRTKKITQNGITEFLWEGERLLGERTADGFRWYLYQP
ETYIPLAVLENGSIYLYECDQVGKPERLKDSAGNIVWSASYDVHGFASIDVEEVRNPLRFQ
GQYFDQETNLHYNLARYYDPKLGRFIQQDPISIAGGINHYQYAVNPIQWIDPTGFLCEEGL

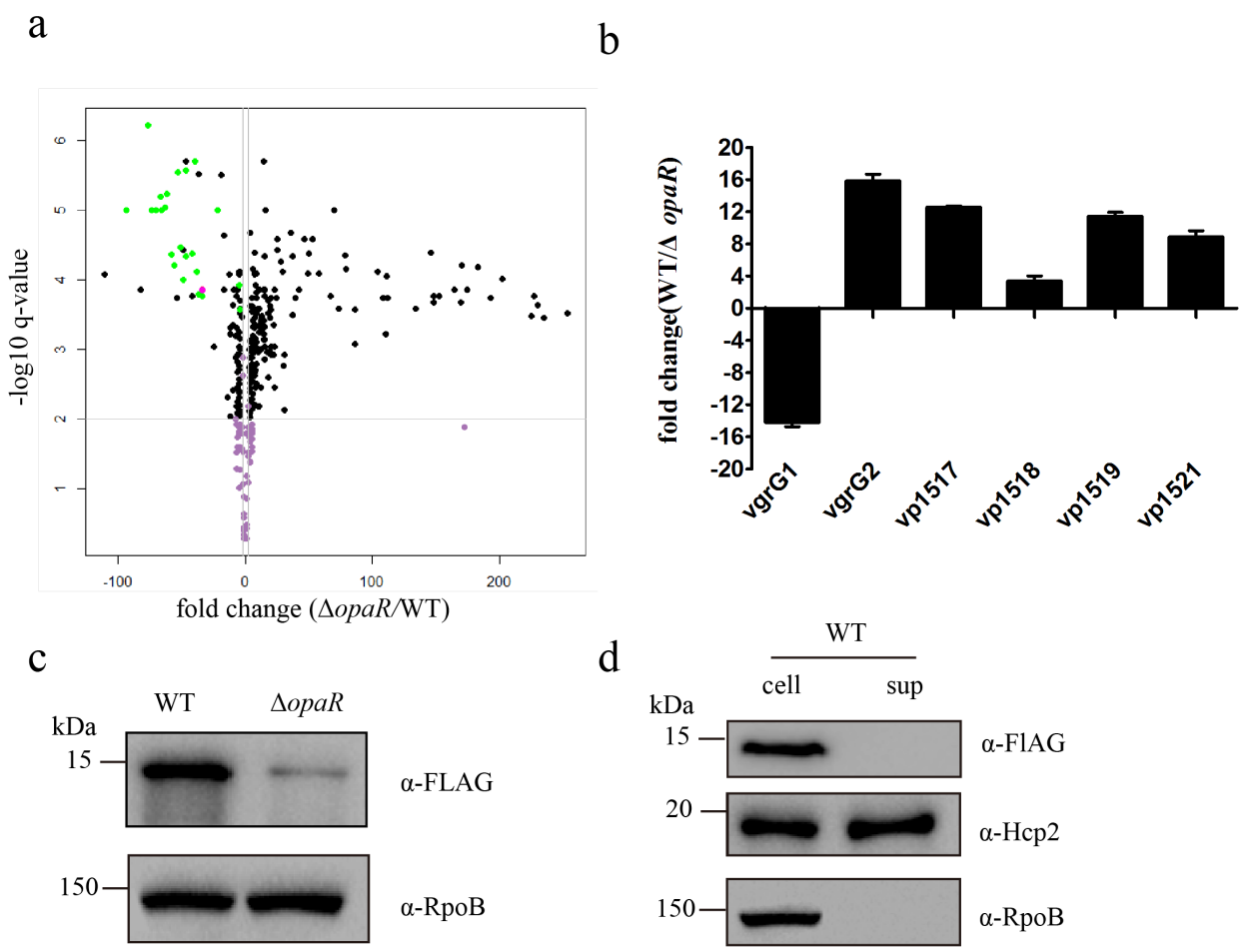
KRLQQMLAEYQAQSDVPQEVCDQILEAAKESSVGEDGVRSQVKIRKPNGKNNIRYEYD

LDHIDCKKNEITFYRHINYSDGSKRKIQYTVGIEGFVDIYDFVNVQKCDAQVYDTKTSKT
VGGRKIINSEFAGKTVTTKGGDVRFDSDGFPDFTPYSKKTVRVIGLTGDMANDVPLAMA
RAKITKYDKSKYVWHHHQDGKTMMLIPKSVHSVRNGGVAHTGGRSVIQHNLLNPNNKL
NYSSPEELV

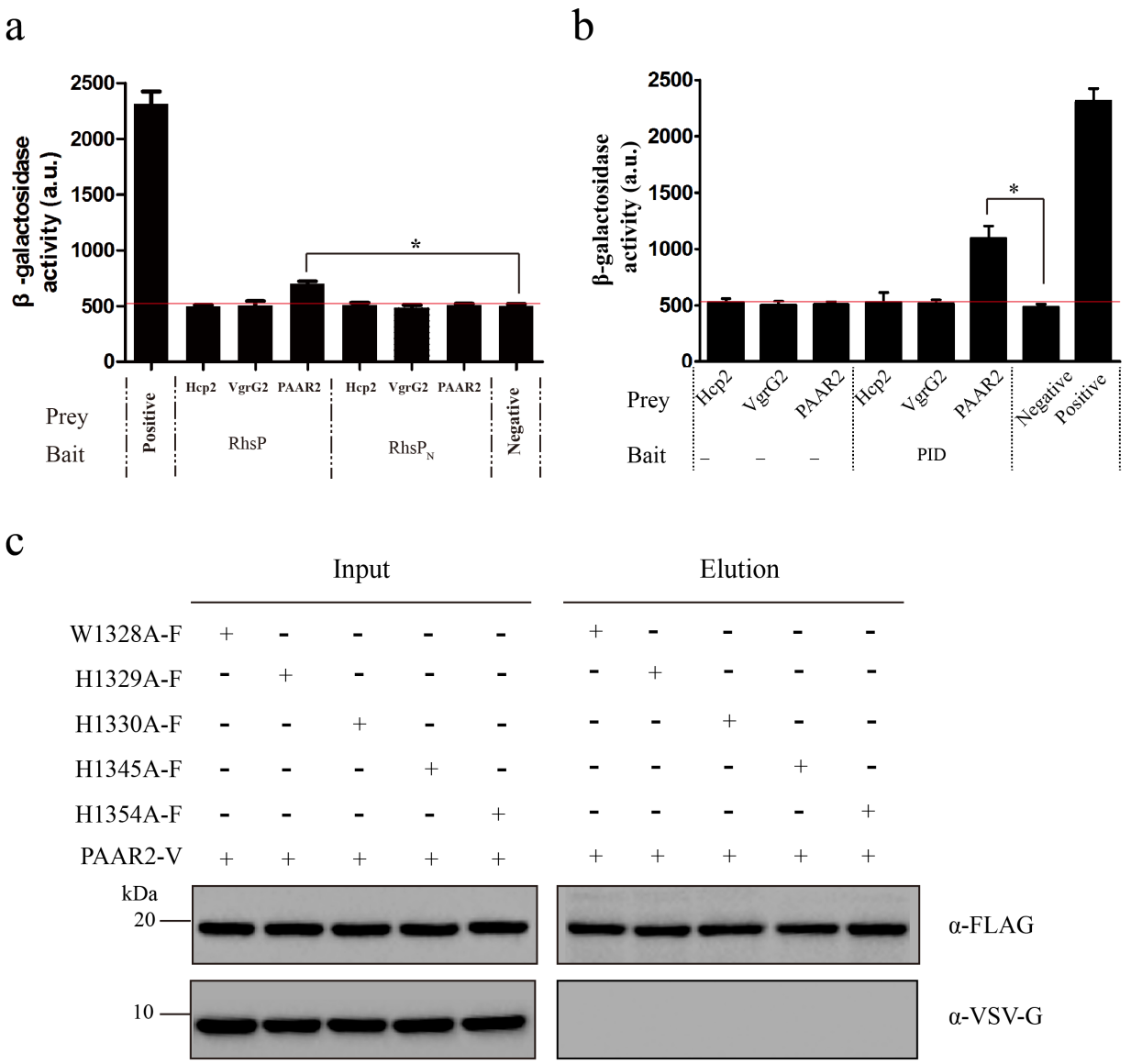
Supplementary Fig. 2. Auto-proteolysis of RhsP. (a) RhsP was self-cleaved and its C-terminal toxin domain was released upon incubation in acidic condition. The pH value of the incubation condition was shown. (b) Sequence annotation of RhsP. Different domains are shown in colours. Green: RHS repeats; Red: intrinsic aspartic protease; Orange: PAAR-interacting domain PID; Blue: WHH domain. The larger font highlighted methionine (M) is the cleavage site.



Supplementary Fig. 3. (a) Deletion of *vp1516* in *V. parahaemolyticu*s RIMD 2210633 has no effect on the secretion of RhsP. FLAG tagged RhsP (RhsP-F) was expressed in Δ*rhsP* or Δ*rhsP*Δ*vp1516* and the secretion of RhsP and its derivatives were examined by Western blot analysis of the total (Cell) and secreted proteins (Sup) by α-FLAG antibody. (b) Deletion of *rhsP* in *V. parahaemolyticu*s RIMD 2210633 did not affect Hcp2 secretion.

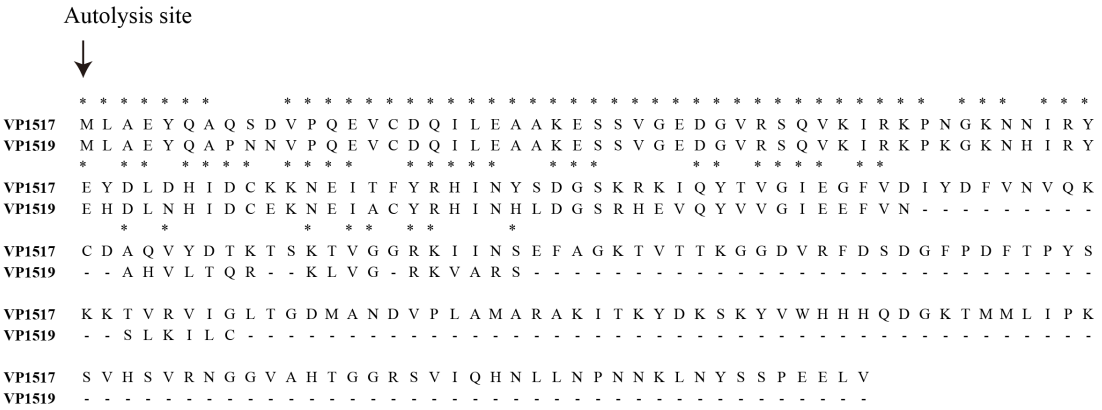


Supplementary Fig. 4. The expression of *rhsP* and *rhsPi* is positively regulated by OpaR. (a) Volcano map visualization of the genes regulated by OpaR based on the microarray data published previously[1]. *rhsP* (megenta dot) and the T6SS2 genes (green dot) are marked. (b) Real-time PCR validation of OpaR regulation on genes in *rhsP* loci. *vgrG1* from T6SS1 and *vgrG2* from T6SS2 were included as controls. (c) Western blot analysis of the expression level of FLAG tagged RhsPi in wild-type *V. parahaemolyticus* and the corresponding Δ*opaR*, both of which contain a chromosomal *rhsPi*::*flag* (*vp1518*::*flag*). (d) RhsPi is not secreted. Western blot analysis of the secretion of the RhsPi in wild-type *V. parahaemolyticus*, which contains a chromosomal *rhsPi*::*flag*.

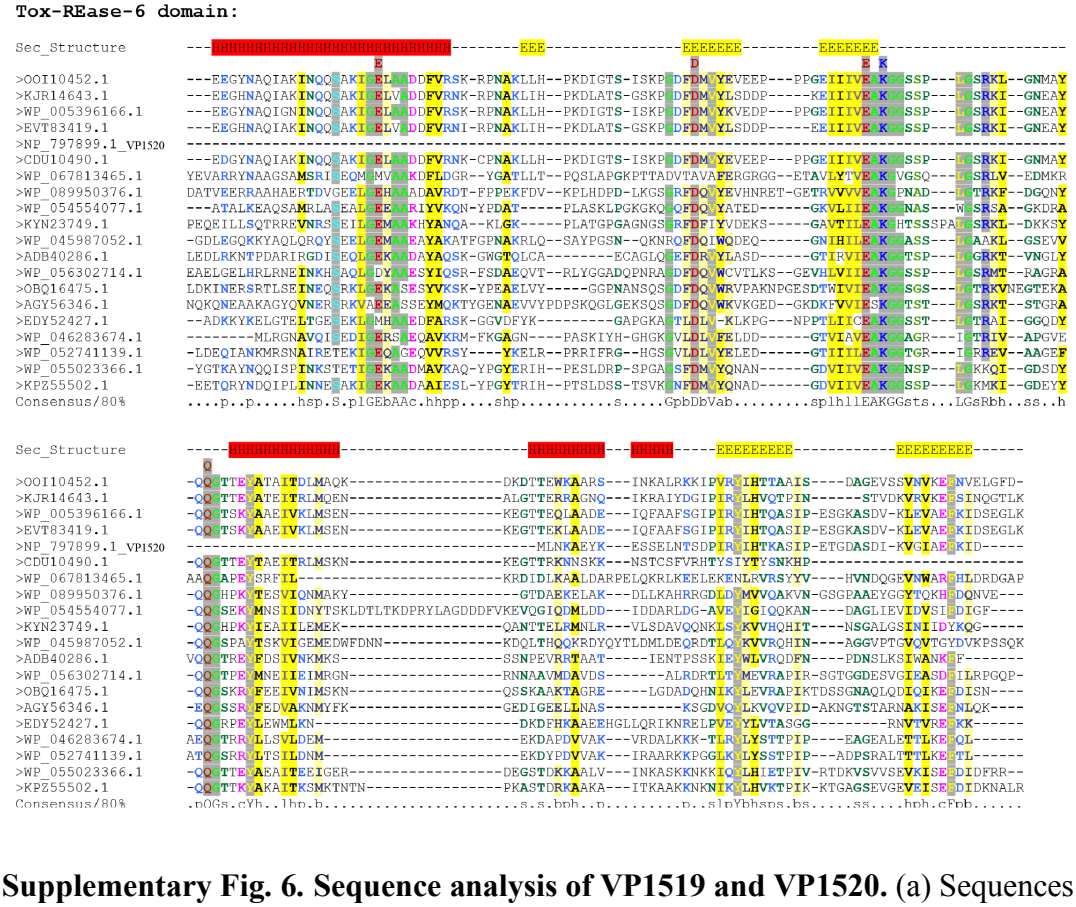


Supplementary Fig. 5. RhsP interacts with PAAR2. (a) Bacterial two-hybrid assay to detect the interaction of full-length RhsP or N-terminal of RhsP ($RhsP_N$) with selected T6SS2 components. (b) Bacterial two-hybrid assay to detect the interaction between PAAR-interacting domain PID (the first 102 amino acids after the auto-proteolysis site) and the components of T6SS puncture device. For (a) and (b), the values represent the means ± s.d. from one representative experiment performed with triplicate samples. Equivalent results were obtained at least three times. Significance was determined by the two-tailed Student *t* test. *$p<0.05$. (c) Co-immunoprecipitation between WHH domain derivatives (different site-directed mutants) and PAAR2. Results showed that various WHH domain derivatives do not interact with PAAR2. WHH domain derivatives and PAAR2 were expressed in *E. coli* DH5α.

a



b



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115 **Supplementary Fig. 6. Sequence analysis of VP1519 and VP1520.** (a) Sequences alignment of
116 PID of VP1517 with VP1519. VP1519 shows high homology with the amino acid sequences of
117 PID in VP1517. (b) VP1520 shows similarity with truncated Tox-REase-6 domain.

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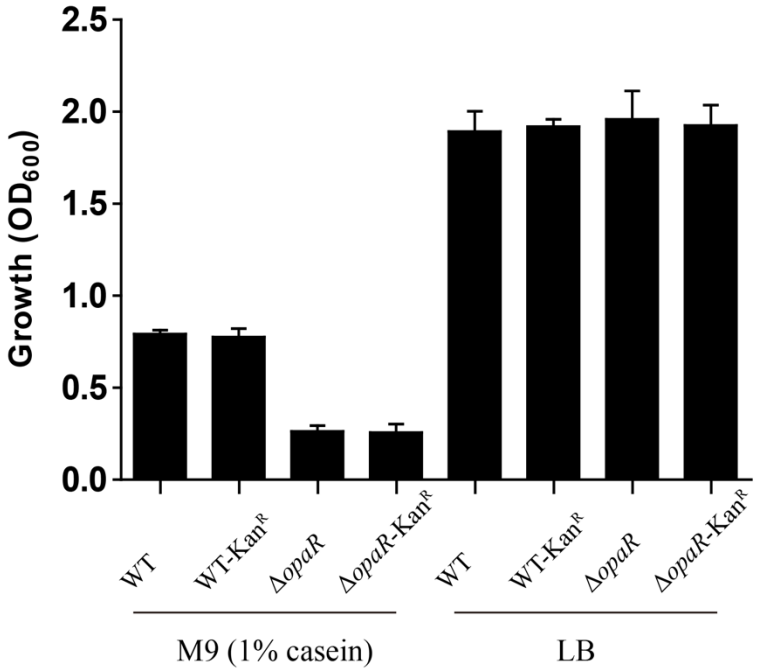
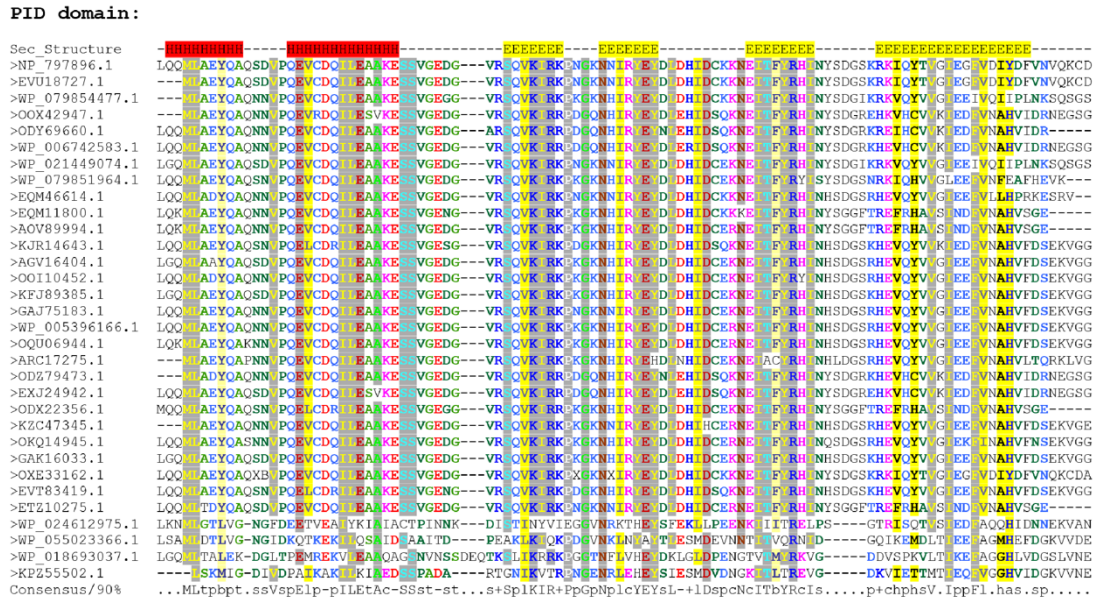


Supplementary Fig. 7. Growth of *V. parahaemolyticus* wild-type (WT) and Δ*opaR* in LB broth and M9 broth minimum media with 1% casein as carbon source. Each strain was inoculated independently and grown overnight in either M9 broth medium containing 1% casein or in LB broth at 37ºC for 16 h, the OD_{600} was then measured. The means of each data set in one representative experiment performed with triplicates was shown. Equivalent results were obtained twice.

a



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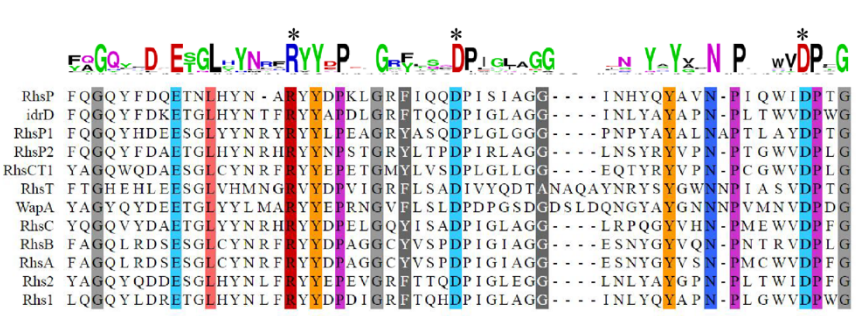
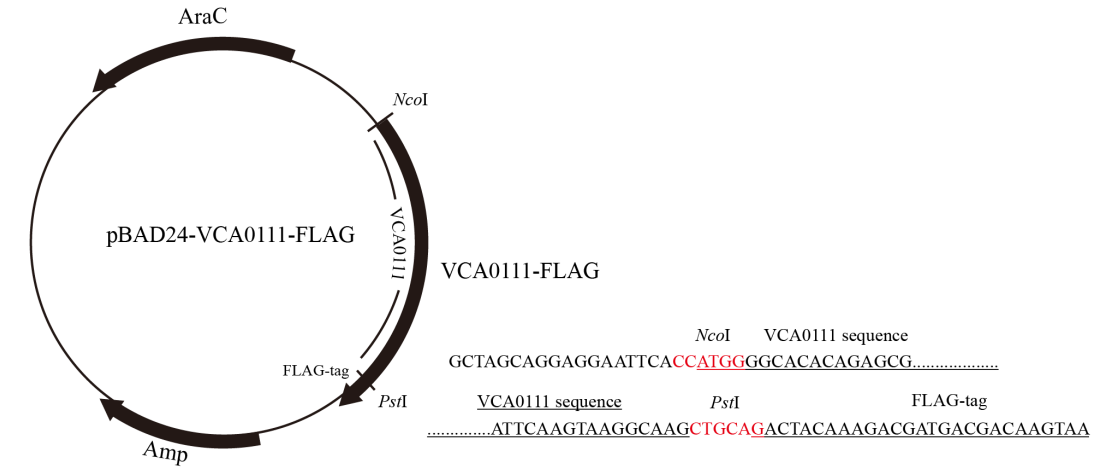


Supplementary Fig. 8. (a) Alignment of amino acid sequences of PID from different pro-effectors identified in Fig. 5a. (b) Alignment of the conserved aspartic protease domain in PAAR-Rhs effectors that have been reported previously[2-9]. Characteristic residues (RDD) are indicated by asterisks.

a



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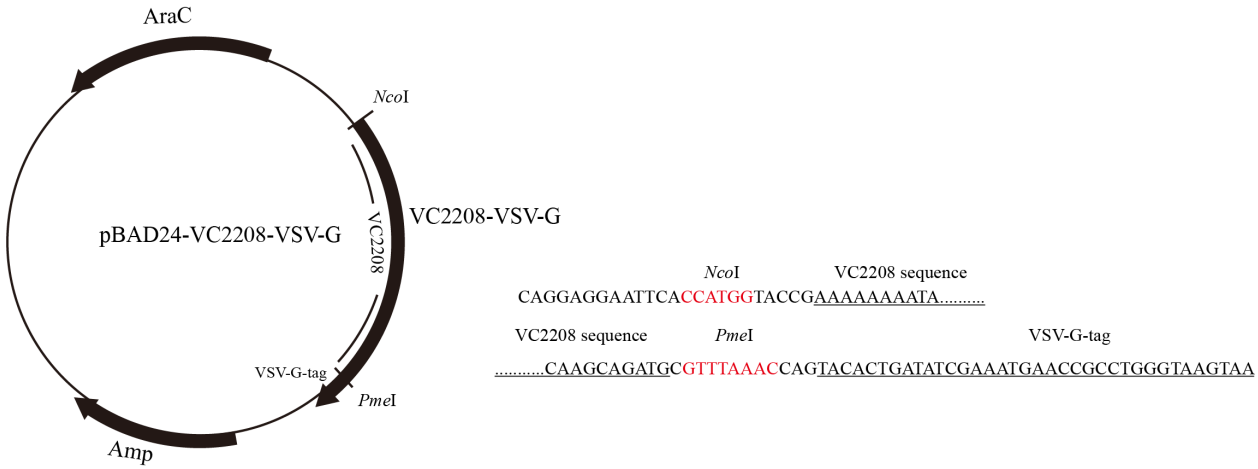
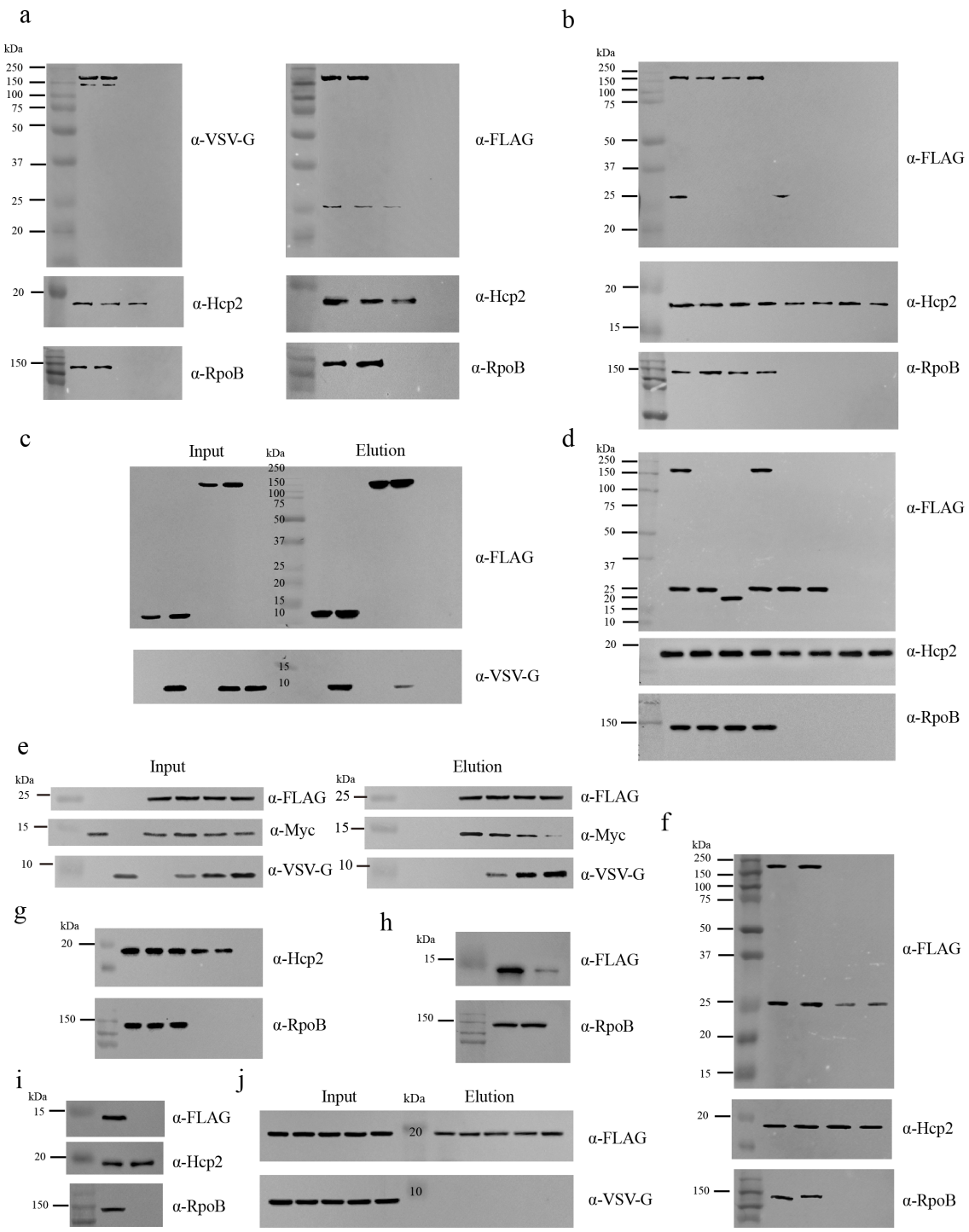


Supplementary Fig. 9. The map and nucleic acids sequences of cloning sites for vector expressing (a) FLAG tag and (b) VSV-G tag**.** Tested gene was amplified by primers listed in Supplementary Table 5 and the PCR product was ligated into *Nco*I/*Pst*I digested pBAD24-VCA0111-FLAG to construct FLAG tagged protein or ligated into *Nco*I/*Pme*I digested pBAD24-VC2208-VSV-G to construct VSV-G tagged protein.



Supplementary Fig. 10. Original figure of western blot analysis for results used in the article. (a) Full picture for Fig. 2e. (b) Full picture for Fig. 3a. (c) Full picture for Fig. 3b. (d) Full picture for Fig. 3c. (e) Full picture for Fig. 4c. (f) Full picture for Supplementary Fig. 3a. (g) Full picture for Supplementary Fig. 3b. (h) Full picture for Supplementary Fig. 4c. (i) Full picture for Supplementary Fig. 4d. (j) Full picture for Supplementary Fig. 5c. All images in this study were acquired with a ChemiDoc imaging system.

Supplementary Tables

Supplementary Table 1. Novel toxins retrieved from *V. parahaemolyticus* RIMD 2210633 genome

Toxin NCBI ID	Gene name	Domain architecture	Operonic structure
NP_797794.1	VP1415	DUF4150+Tox-AHH	OmpA->DUF4150+Tox-AHH*->Nimm11->Nimm11->Nimm11->Nimm11->Nimm11->
NP_800773.1	VPA1263	LysM+PyocinS+Tox-ColE7	LysM+PyocinS+Tox-ColE7*->
NP_800280.1	VPA0770	Tox-RES	DUF2384->Tox-RES*->
NP_797900.1	VP1517	RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+Tox-WHH	RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+RHS+Tox-WHH*->Imm-SUKH->?-?->Nimm49*->Nimm49->

Supplementary Table 2. Bacterial strains and plasmids used in this study

Bacterial strains/plasmids	Description	Source
Vibrio parahaemolyticus		
RIMD 2210633	Wild-type *V. parahaemolyticus*, Amp^R	[10]
Δ*rhsP*	RIMD 2210633, in-frame deletion of *rhsP*	This study
Δ*rhsP*Δ*paar2*	RIMD 2210633, in-frame deletion of *rhsP* and *paar2*	This study
Δ*rhsP*Δ*clpV2*	RIMD 2210633, in-frame deletion of *rhsP* and *clpV2*	This study
Δ*rhsP* + *V-rhsP-F*	Δ*rhsP* complemented with pBAD33- *V-RhsP-F*	This study
Δ*rhsP*Δ*clpV2* + *V-rhsP-F*	Δ*rhsP*Δ*clpV2* complemented with pBAD33-*V-RhsP-F*	This study
Δ*rhsP* + *rhsP-F*	Δ*rhsP* complemented with pBAD33-*RhsP-F*	This study
Δ*rhsP* + $rhsP_C$-*F*	Δ*rhsP* complemented with pBAD33-$rhsP_C$-*F*	This study
Δ*rhsP* + WHH domain-F	Δ*rhsP* complemented with pBAD33-WHH domain-F	This study
Δ*rhsP*Δ*paar2* + *rhsP-F*	Δ*rhsP*Δ*paar2* complemented with pBAD33-RhsP-F	This study
Δ*rhsP*Δ*rhsPi*	RIMD 2210633, in-frame deletion of *rhsP* and *rhsPi*	This study
Δ*clpV2*	RIMD 2210633, in-frame deletion of *clpV2*	This study
Δ*clpV2* + *clpV2*	Δ*clpV2* complemented with pBAD33-*clpV2*	This study
Δ*opaR*	RIMD 2210633, in-frame deletion of *opaR* (*vp2516*)	This study
Δ*opaR*-Kan^R	Δ*opaR* complemented with pBBR1MCS2, Kan^R	This study
Δ*opaR* -Chl^R	Δ*opaR* containing pBAD33, Chl^R	This study
Δ*opaR* + *rhsPi*	Δ*rhsP* complemented with pBAD33-*rhsPi*	This study
Δ*rhsP*Δ*vp1516*	RIMD 2210633, in-frame deletion of *rhsP* and *vp1516*	This study
Δ*rhsP*Δ*vp1516* + *rhsP-F*	Δ*rhsP*Δ*vp1516* complemented with pBAD33-RhsP-F	This study
E. coli		
DH5αλ*pir*	F- φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*) *LAMpir* U169 *endA1 recA1 hsdR17*(rk-,mk+) supE44λ- *thi* -1 *gyr*A96 *rel*A1 *pho*A	Invitrogen
BL21(DE3)	F- *dcm ompT hsdS gal* λ (DE3)	Novagen
MFD*pir*	MG1655 RP4-2-Tc:: Mu1::*aac(3)IV- aphA- nic*35-Mu2::*zeo dapA*::(*erm-pir*) *recA*	[11]
Plasmid		

pET28a	cloning vector, PT7, Kan^R	Invitrogen
pET28a-*rhsP*	pET28a expressing RhsP tagged with 6×His at C-terminal	This study
pET28a- R1092A	pET28a expressing RhsP mutant of R1092A tagged with 6×His at C-terminal	This study
pET28a- D1105A	pET28a expressing RhsP mutant of R1105A tagged with 6×His at C-terminal	This study
pET28a- D1127A	pET28a expressing RhsP mutant of R1127A tagged with 6×His at C-terminal	This study
pBAD24	cloning vector, arabinose induced, Amp^R	Invitrogen
pBAD24-VCA0111-FLAG	pBAD24 expressing VCA0111-FLAG	This study
pBAD24-VC2208-VSV-G	pBAD24 expressing VC2208-VSV-G	This study
pBAD24-*rhsP-F*	pBAD24 expressing RhsP tagged with FLAG epitope at C-terminal	This study
pBAD24-WHH domain-F	pBAD24 expressing WHH domain tagged with FLAG epitope at C-terminal	This study
pBAD24-W1328A	pBAD24 expressing WHH domain with point mutation of W1328A tagged with FLAG epitope at C-terminal	This study
pBAD24-H1329A	pBAD24 expressing WHH domain with point mutation of W1329A tagged with FLAG epitope at C-terminal	This study
pBAD24-H1330A	pBAD24 expressing WHH domain with point mutation of W1330A tagged with FLAG epitope at C-terminal	This study
pBAD24-H1345A	pBAD24 expressing WHH domain with point mutation of W1345A tagged with FLAG epitope at C-terminal	This study
pBAD24-H1354A	pBAD24 expressing WHH domain with point mutation of W1354A tagged with FLAG epitope at C-terminal	This study
pBAD24-PID-F	pBAD24 expressing PID (from M1143 to D1244 of RhsP) tagged with FLAG epitope at C-terminal	This study
pBAD24-*paar2-V*	pBAD24 expressing PAAR2 tagged with VSV-G epitope at C-terminal	This study
pBAD33	cloning vector, arabinose induced, Chl^R	Invitrogen
pBAD33-*V-rhsP-F*	pBAD33 expressing RhsP tagged with FLAG epitope at C-terminal and VSV-G at its N-terminal	This study
pBAD33-*rhsP-F*	pBAD33 expressing RhsP tagged with FLAG epitope at C-terminal	This study

pBAD33-*rhsP-F* (R1092A)	pBAD33 expressing point mutated RhsP (R1092A) tagged with FLAG epitope at C-terminal	This study
pBAD33- *rhsP-F* (D1105A)	pBAD33 expressing point mutated RhsP (D1105A) tagged with FLAG epitope at C-terminal	This study
pBAD33- *rhsP-F* (D1127A)	pBAD33 expressing point mutated RhsP (D1127A) tagged with FLAG epitope at C-terminal	This study
pBAD33-*rhsP$_C$-F*	pBAD33 expressing RhsP$_C$ tagged with FLAG epitope at C-terminal	This study
pBAD33-WHH domain-F	pBAD33 expressing WHH domain tagged with FLAG epitope at C-terminal	This study
pBAD33-*clpV2*	pBAD33 expressing ClpV2	This study
pBAD33-*rhsPi* (*vp1518*)	*Vp1518* cloned into pBAD33	This study
pCX340	pBBR322 derivative, IPTG induced, Tet^R	[12]
pCX340-*rhsPi* (*vp1518*)	pCX340 with *vp1518* including stop codon	This study
pCX340-*vp1519*	pCX340 with *vp1519* including stop codon	This study
pCX340-*vp1520*	pCX340 with *vp1520* including stop codon	This study
pCX340-*rhsPi-M*	pCX340 expressing RhsPi (VP1518) tagged with Myc epitope at C-terminal	This study
pACλCI	Plasmid for bacterial two-hybrid; Encodes λCI (residues 1-236) under the control of lacUV5 promoter; Chl^R	[13]
pBRα	Plasmid for bacterial two-hybrid; Encodes the full-length α subunit of RNAP under the control of tandem *placUV5* and *plpp* promoters; Carb^R	[13]
pACλCI-β-flap (831–1057)	Positive control for bacterial two-hybrid; Encodes residues 1-248 of α fused by a three-alanine linker residues 831–1057 of the β subunit of *E. coli* RNAP under the control of tandem p*lacUV5* and p*lpp* promoters; confers resistance to Carb; used to generate *Not*I/*Bam*HI-digested backbone to construct various α fusions; Chl^R	[13]
pBRα –β-flap (831–1057)	Positive control for bacterial two-hybrid; Encodes residues 1-248 of α fused by a three-alanine-linker residues 831–1057 of the β subunit of *E. coli* RNAP under the control of tandem *placUV5* and *plpp* promoters; used to generate *Not*I/*Bam*HI-digested backbone to construct various α fusions; Carb^R	[13]
pAC-*hcp2*	pACλcI with *hcp2*	This study

pAC-*paar2*	pACλcI with *paar2*	This study
pAC-*vgrG2*	pACλcI with *vgrG2*	This study
pBR-*rhsP*	pBRα with *rhsP*	This study
pBR-PID	pBRα with fragment encoding PID	This study
pBR- $rhsP_N$	pBRα with $rhsP_N$	This study

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Supplementary Table 3. Buffers formula for auto-proteolysis testing of RhsP and its derivatives

pH	0.1M acetic acid (ml)	0.1 sodium acetate (tri-hydrate) (ml)
3	982.3	17.7
4	847	153
5	357	643
6	52.2	947.8

Supplementary Table 4. Primers used in this study

Name	Sequence	Destination
Primers for mutant construction		
VP1517up-F	AAAAAGGATCGATCCTCTAGATTTGGATTGCGTATATCC	For construction of deletion mutant of Δ*rhsP*
VP1517up-R	ATCAACTCCAGTTGTAACGAGTGTGTCC	
VP1517down-F	GTTACAACTGGAGTTGATATATGATTAGCC	
VP1517down-R	ATCGCATGCGGTACCTCTAGAATTCAGCACAAGATCTTT	
VP1517-18 cluster up -F	AAAAAGGATCGATCCTCTAGATTTGGATTGCGTATATCC	For construction of deletion mutant of Δ*rhsP*Δ*rhsPi*
VP1517-1518 cluster up-R	TTGTCAAAGAGTTGTAACGAGTGTG	
VP1517-18 cluter down -F	GTTACAACTCTTTGACAAAACAGGG	
VP1517-1518 cluster down-R	ATCGCATGCGGTACCTCTAGATACAAATTTGTTCGTCTTCA	
VP1518FLAG-up-F	AAAAAGGATCGATCCTCTAGACTATTCAGATGGTAGTAAAC	To insert Flag tag following RhsPi (VP1518)
VP1518FLAG-up-R	TTACTTGTCGTCATCGTCTTTGTAGTCAGATTCTAGAAGCTTTTCCA	
VP1518FLAG-down-F	GACGATGACGACAAGTAACTTTGACAAAACAGGGAAGA	
VP1518FLAG-down-R	ATCGCATGCGGTACCTCTAGACAAATTTGTTCGTCTTCATG	
VPA1025up-F	AAAAAGGATCGATCCTCTAGACACGAAGATAAATCGCGA	For construction of deletion mutant of Δ*paar2* (VPA1025)
VPA1025up-R	ACCGACGAGTCTAAGTTCCCTCTTAATTCA	
VPA1025down-F	GAACTTAGACTCGTCGGTGTACCGACCGTC	
VPA1025down-R	ATCGCATGCGGTACCTCTAGAATGTGGTTTGTGTTGATT	
VPA1028up-F	AAAAAGGATCGATCCTCTAGAGTTACTTCTTTACCC	For construction of deletion mutant of *clpV2* (VPA1028)
VPA1028up-R	TTTAAAGGAAACATACAAGTTTAACTCTGT	
VPA1028down-F	ACAGAGTTAAACTTGTATGTTTCCTTTAAA	
VPA1028down-R	ATCGCATGCGGTACCTCTAGAACCGCGAGCTAAAGC	
VP2516up-F	AAAAAGGATCGATCCTCTAGAAACGCCCATTTATATGGG	For construction of deletion mutant of Δ*opaR* (VP2516)

VP2516up-R	AAATCTGAGCTATCCATTTTCCTTGCCATT	
VP2516down-F	AAAATGGATAGCTCAGATTTGAACACGAAC	
VP2516down-R	ATCGCATGCGGTACCTCTAGACGTTAAGGTTTACACCAA	
Primers for constructs for complementation		
VP1517-WHH-FLAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGGCTCAAGTATATGATAC	For cloning variable length of *RhsP* into pBAD33
VP1517C-FlAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGTTCCTTTGTGAAGAAGG	
VP1517-FLAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGATTCCTCAATTTGTTAT	
VSVG-VP1517-FLAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGTATACAGACATAGAGATGAACCGACTTGGAAAGATTCCTCAATTTGTTAT	
VP1517FLAGcom-R	GATCCCCGGGTACCGAGCTCTTACTTGTCGTCATCGTCTT	
VP1517-FLAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGATTCCTCAATTTGTTAT	For construction of RhsP mutant R1092A in pBAD33
VP1517-R1092A-R	GTCGTAATA**CGC**AGCGAGGTT	
VP1517-R1092A-F	AACCTCGCT**GCG**TATTACGAC	
VP1517FLAGcom-R	GATCCCCGGGTACCGAGCTCTTACTTGTCGTCATCGTCTT	
VP1517-FLAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGATTCCTCAATTTGTTAT	For construction of RhsP mutant D1105A in pBAD33
VP1517-D1105A-R	AGATATCGG**GGC**TTGTTGGAT	
VP1517-D1105A-F	ATCCAACAA**GCC**CCGATATCT	
VP1517FLAGcom-R	GATCCCCGGGTACCGAGCTCTTACTTGTCGTCATCGTCTT	
VP1517-FLAGcom-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGATTCCTCAATTTGTTAT	For construction of RhsP mutant D1127A in pBAD33
VP1517-D1127A-R	ACCTGTTGG**GGC**AATCCACTG	
VP1517-D1127A-F	CAGTGGATT**GCC**CCAACAGGT	
VP1517FLAGcom-R	GATCCCCGGGTACCGAGCTCTTACTTGTCGTCATCGTCTT	
VP1518com-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGATTAGCCTTTCAGATATTG	For construction of RhsPi in pBAD33
VP1518com-R	GATCCCCGGGTACCGAGCTCTTAAGATTCTAGAAGCTTTTCC	
VPA1028com-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC***ATGGCTTCATTATCA	For construction of ClpV2 in pBAD33

VPA1028com-R	GATCCCCGGGTACCGAGCTCTTAAATAAGTGAGACTTCT	
VP2516com-F	GGGCTAGCGAATTCGAGCTC***AGGAGGAATTCACC*** ATGGACTCAATTGCA	For cloning *opaR* into plasmid pBAD33
VP2516com-R	GATCCCCGGGTACCGAGCTCTTAGTGTTCGCGATTGTAG	
*Bold: Alanine substituted site; Bold and Italic: ribosome-binding site		
Primers for constructs used in Co-IP		
pBAD24-vpa1025VSVG-F	GAGGAATTCACCATGGGTAAACCAGCAGCAGTGAT	For cloning *paar2* into plasmid pBAD24-VC2208-VSV-G plasmid
pBAD24-VPA1025VSVG-R	GATATCAGTGTACTGGTTTAAACGTCCCCCGATGAGGACGGTCG	
pBAD24-PID-FLAG-F	GAGGAATTCACCATGTTCCTTTGTGAAGAAGG	For cloning PID into plasmid pBAD24-VCA0111-FLAG plasmid
pBAD24-PID-FLAG-R	ATCGTCTTTGTAGTCGTCACATTTTTGAACATT	
Primers for expressing toxin and antitoxin		
pBAD24-1517FLAG-F	GAGGAATTCACCATGATTCCTCAATTTGTTAT	For cloning *rhsP* and WHH domain into plasmid pBAD24-VCA0111-FLAG
pBAD24-1517CT-FLAG-F	GAGGAATTCACCATGGCTCAAGTATATGATACTAA	
pPAD24-1517CT-FLAG-R	ATCGTCTTTGTAGTCTACGAGCTCTTCTGGAGACGAGTA	
pBAD24-1517CT-FLAG-F	GAGGAATTCACCATGGCTCAAGTATATGATACTAA	Overlapping primers to amplify mutated WHH(W1328A)
WHH-W1328A-R	CTTGATGATGATG**CGC**AACAT	
WHH-W1328A-F	ATGTT**GCG**CATCATCATCAAG	
pPAD24-1517CT-FLAG-R	ATCGTCTTTGTAGTCTACGAGCTCTTCTGGAGACGAGTA	
pBAD24-1517CT-FLAG-F	GAGGAATTCACCATGGCTCAAGTATATGATACTAA	Overlapping primers to amplify mutated WHH (H1329A)
WHH-H1329A-R	CATCTTGATGATG**TGC**CCAAA	
WHH-H1329A-F	TTTGG**GCA**CATCATCAAGATG	
pPAD24-1517CT-FLAG-R	ATCGTCTTTGTAGTCTACGAGCTCTTCTGGAGACGAGTA	
pBAD24-1517CT-FLAG-F	GAGGAATTCACCATGGCTCAAGTATATGATACTAA	Overlapping primers to amplify mutated WHH (H1330A)
WHH-H1330A-R	ACCATCTTGATG**GGC**ATGCCA	
WHH-H1330A-F	TGGCAT**GCC**CATCAAGATGGT	
pPAD24-1517CT-F	ATCGTCTTTGTAGTCTACGAGCTCTTCTGGAGACG	

LAG-R	AGTA	
pBAD24-1517CT-F LAG-F	GAGGAATTCACCATGGCTCAAGTATATGATACTAA	Overlapping primers to amplify mutated WHH (H1345A)
WHH-H1345A-R	TTCTAACTGA**GGC**CACACTTT	
WHH-H1345A-F	AAAGTGTG**GCC**TCAGTTAGAA	
pPAD24-1517CT-F LAG-R	ATCGTCTTTGTAGTCTACGAGCTCTTCTGGAGACG AGTA	
pBAD24-1517CT-F LAG-F	GAGGAATTCACCATGGCTCAAGTATATGATACTAA	Overlapping primers to amplify mutated WHH (H1354A)
WHH-H1354A-R	CTACCACCTGT**GGC**AGCTACA	
WHH-H1354A-F	TGTAGCT**GCC**ACAGGTGGTAG	
pPAD24-1517CT-F LAG-R	ATCGTCTTTGTAGTCTACGAGCTCTTCTGGAGACG AGTA	
	*Bold: Alanine substituted site	
pCX340-1518-F	CATATGGGAAGCTTGGGTACCATGATTAGCCTTTC AGATATTG	For cloning *rhsPi* into plasmid pCX340 to express RhsPi-Myc
pCX340-1518-R	AGCGTTTCTGGGTGCGAATTCTTAAGATTCTAGAA GCTTTTCC	
pCX340-1518myc-R	AGCGTTTCTGGGTGCGAATTCTTACAGGTCTTCTT CAGAGATCAGTTTCTGTTCAGATTCTAGAAGCTTT TCC	
pCX340-1519-F	CATATGGGAAGCTTGGGTACCATGCTGGCAGAGTA TCAGGCGC	For cloning *vp1519* into plasmid pCX340
pCX340-1519-R	AGCGTTTCTGGGTGCGAATTCTTAGCACAAGATCT TTAGTGAAG	
pCX340-1520-F	CATATGGGAAGCTTGGGTACCATGCTGAATAAAGC AGAATATAA	For cloning *vp1520* into plasmid pCX340
pCX340-1520-R	AGCGTTTCTGGGTGCGAATTCTTAATCAATTTTAA ACTCAGCTA	
pBAD24-WP021FL AG-F	GAGGAATTCACCATGTTGAATAAAAGTCAA	For cloning the fragment of the AHH domain in strain WP-021449074 into pBAD24 plasmid
pBAD24-WP021FL AG-R	ATCGTCTTTGTAGTCCTACTTCCTACTTTT	
pBAD24-WP005FL AG-F	GAGGAATTCACCATGGTTGGTGGGGAGAAA	For cloning the fragment of the REase-6 domain in strain WP-005396166 into pBAD24 plasmid
pBAD24-WP005FL AG-R	ATCGTCTTTGTAGTCCTTTAGTCCTTC	
pBAD24-WP006-F	GAGGAATTCACCATGAATGAAGGATCTGGT	For cloning the fragment of the AHH domain in strain WP-006742583 into
pBAD24-WP006-R	ATCGTCTTTGTAGTCTCTCCTCACAGC	

		pBAD24 plasmid
pCX340-antiWP012-F	CATATGGGAAGCTTGGGTACCATGACACTTAAAATTTGG	For cloning the fragment of the immunity protein of the AHH domain in strain WP-021449074 into pCX340 plasmid
pCX340-antiWP012-R	AGCGTTTCTGGGTGCGAATTCTTAAAAAGGACGCCATTC	
pCX340-WP005FLAG-F	CATATGGGAAGCTTGGGTACCATGCAAAGCAAAGAATCG	For cloning the fragment of the immunity protein of the REase-6 domain in strain WP-005396166 into pCX340 plasmid
pCX340-WP005FLAG-R	AGCGTTTCTGGGTGCGAATTCTTATCGAGTGATCTTCCC	
pCX340-WP006-F	CATATGGGAAGCTTGGGTACCATGGCAGGTGAGCATAGC	For cloning the fragment of the immunity protein of the AHH domain in strain WP-006742583 into pCX340 plasmid
pCX340-WP006-R	AGCGTTTCTGGGTGCGAATTCTTAAGCGTGCCTATTGTG	
Primer for pET28a cloning		
28a-vp1517-F	TAAGAAGGAGATATACCATGATTCCTCAATTTGTTAT	For cloning *rhsP* into pET28a plasmid
28a-vp1517-R	GTGGTGGTGGTGGTGTACGAGCTCTTCTGGAGACGA	
28a-vp1517-F	TAAGAAGGAGATATACCATGATTCCTCAATTTGTTAT	For construction of RhsP mutant R1092A in pET28a
VP1517-R1092A-R	GTCGTAATA**CGC**AGCGAGGTT	
VP1517-R1092A-F	AACCTCGCT**GCG**TATTACGAC	
28a-vp1517-R	GTGGTGGTGGTGGTGTACGAGCTCTTCTGGAGACGA	
28a-vp1517-F	TAAGAAGGAGATATACCATGATTCCTCAATTTGTTAT	For construction of RhsP mutant D1105A in pET28a
VP1517-D1105A-R	AGATATCGG**GGC**TTGTTGGAT	
VP1517-D1105A-F	ATCCAACAA**GCC**CCGATATCT	
28a-vp1517-R	GTGGTGGTGGTGGTGTACGAGCTCTTCTGGAGACGA	
28a-vp1517-F	TAAGAAGGAGATATACCATGATTCCTCAATTTGTTAT	For construction of RhsP mutant D1127A in pET28a
VP1517-D1127A-R	ACCTGTTGG**GGC**AATCCACTG	
VP1517-D1127A-F	CAGTGGATT**GCC**CCAACAGGT	
28a-vp1517-R	GTGGTGGTGGTGGTGTACGAGCTCTTCTGGAGACGA	
Primers for bacterial two hybrid		
pAC-Hcp2-F	ACGTTTGGCGCGGCCGCTATGCAGTCTAATACGTATCT	For cloning Hcp2 into pACλCI plasmid
pAC-Hcp2-R	CCTGCGATGCGGATCCTTACATTTGTTGACCTTTA	

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pAC-VgrG2-F	ACGTTTGGCGCGGCCGCTATGAAAAAAGCAAGTCAAGA	For cloning VgrG2 into pACλCI plasmid
pAC-VgrG2-R	CCTGCGATGCGGATCCTTAATTCAAAGAGATTTGTC	
pAC-PAAR2-F	ACGTTTGGCGCGGCCGCTATGGGTAAACCAGCAGCAGT	For cloning PAAR2 into pACλCI plasmid
pAC-PAAR2-R	CCTGCGATGCGGATCCCTATCCCCCGATGAGGACGG	
pBR-RhsP-F	AAACCAGAGGCGGCCGCTATGATTCCTCAATTTGTTAT	For cloning RhsP into pBRα plasmid
pBR-RhsP-R	CCGGCGTAGAGGATCCTCATACGAGCTCTTCTGGAG	
		For cloning $RhsP_N$ into pBRα plasmid
pBR-$RhsP_N$-R	CCGGCGTAGAGGATCCACCRGTTGGGTCAATCCACTGG	
pBR-PID-F	AAACCAGAGGCGGCCGCTATGTTCCTTTGTGAAGAAGG	For cloning PID into pBRα plasmid
pBR-PID-R	CCGGCGTAGAGGATCCTACGTCACATTTTTGAACATT	
Primers for Real time PCR		
RT-vp-vgrG1-F	TGCCGATGATAACCAAACAG	For RT-PCR to targeting *vgrG1*
RT-vp-vgrG1-R	CCACCTTTCACGACGACTTT	
RT-vp-vgrG2-F	TAAAGGTCTTCAAACAGCCA	For RT-PCR to targeting *vgrG2*
RT-vp-vgrG2-R	TTGCCCTACCCGAGGATGAA	
RT-vp1517-F	ATTAACCATTACCAATATGC	For RT-PCR to targeting *rhsP*
RT-vp1517-R	GCGAGCGAACGCCATCTTCT	
RT-vp1518-F	GCCTTTCAGATATTGAAAAT	For RT-PCR to targeting *rhsPi*
RT-vp1518-R	ATCCAAATACTCCTCATAGT	
RT-vp1519-F	TCCGTCGGAGAAGATGGTGT	For RT-PCR to targeting *vp1519*
RT-vp1519-R	GCGCATTTACAAATTCCTCA	
RT-vp1521-F	AATTGTGCGTGATAGACCAA	For RT-PCR to targeting *vp1521*
RT-vp1521-R	CCAGTTATCCCCTTGATCTA	
RT-16s-F	ACTCCTACGGGAGGCAGCAG	For RT-PCR to targeting 16srDNA
RT-16s-R	ATTACCGCGGCTGCTGG	

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