



Article

HrpL Regulon of Bacterial Pathogen of Woody Host *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335

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Supplementary Material

Figure S1. HrpL-dependent expression of HrpL regulon genes by RT-qPCR. Quantitative reverse-transcription polymerase chain reaction with the indicated *P. savastanoi* pv. *savastanoi* NCPPB 3335 genes for its $\Delta hrpL$ mutant vs. wild-type (WT) strain at 6 h after transfer to Hrp-inducing medium. Fold change (fc) was calculated after normalization using the *gyrA* gene as an internal control. Results represent the means for two biological replicates. Error bars represent the standard deviation. Asterisks indicate a significantly different value from one, established by a T-Student test ($P < 0.05$), which indicate a significant decrease in the expression of these genes in the $\Delta hrpL$ mutant compared with WT strain.

Figure S2. Gene expression analysis of biological replicates used in RNA-seq study. The boxplot displays distribution of normalized expression values (\log_{10} FPKM) among the two biological replicates of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 and its $\Delta hrpL$ mutant.

Table S1. Strains used in this study.

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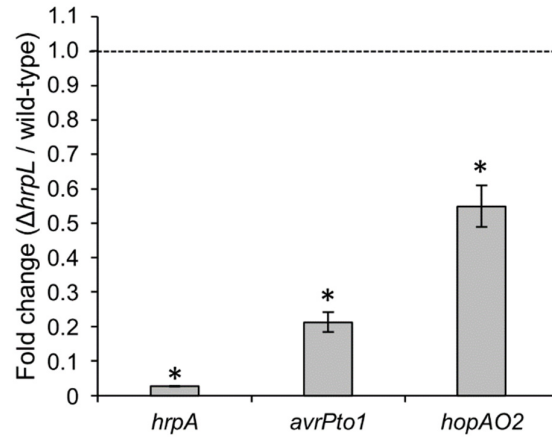


Figure S1. HrpL-dependent expression of HrpL regulon genes by RT-qPCR. Quantitative reverse-transcription polymerase chain reaction with the indicated *P. savastanoi* pv. *savastanoi* NCPPB 3335 genes for its $\Delta hrpL$ mutant vs. wild-type (WT) strain at 6 h after transfer to Hrp-inducing medium. Fold change (fc) was calculated after normalization using the *gyrA* gene as an internal control. Results represent the means for two biological replicates. Error bars represent the standard deviation. Asterisks indicate a significantly different value from one, established by a T-Student test ($P < 0.05$), which indicate a significant decrease in the expression of these genes in the $\Delta hrpL$ mutant compared with WT strain.

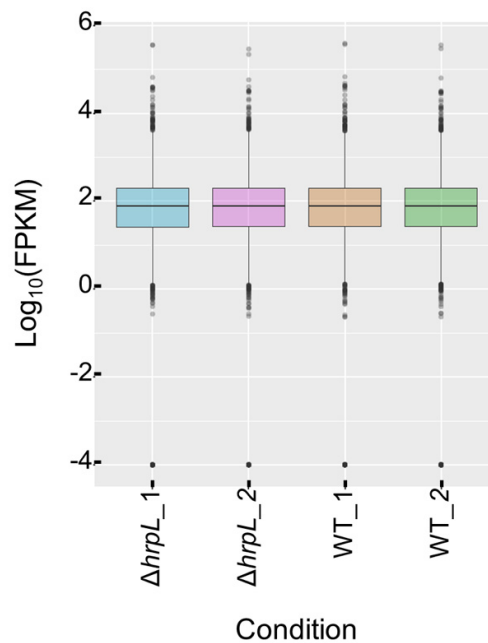


Figure S2. Gene expression analysis of biological replicates used in RNA-seq study. The boxplot displays distribution of normalized expression values (log₁₀ FPKM) among the two biological replicates of *P. savastanoi* pv. *savastanoi* NCPPB 3335 and its $\Delta hrpL$ mutant.

Table S1. Strains used in this study.

Strain	Main features	Reference or source
<i>Escherichia coli</i>		
DH5α	<i>F</i> -, ϕ 80 Δ lacZ M15, (<i>lacZYA-argF</i>) U169, <i>deoR</i> , <i>recA1</i> , <i>endA</i> , <i>hsdR17</i> (<i>rk</i> - <i>mk</i> -), <i>phoA</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	[1]
GM2929	<i>F</i> -, <i>ara-14</i> , <i>leuB6</i> , <i>thi-1</i> , <i>tonA31</i> , <i>lacY1</i> , <i>tsx-78</i> , <i>galK2</i> , <i>galT22</i> , <i>glnV44</i> , <i>hisG4</i> , <i>rpsL136</i> , <i>xyl-5</i> , <i>mtl-1</i> , <i>dam13::Tn9</i> , <i>dcm-6</i> , <i>mcrB1</i> , <i>hsdR2</i> , <i>mcrA</i> , <i>recF143</i> (Sp ^R , Cm ^R)	[2]
<i>Pseudomonas</i>		
<i>P. savastanoi</i> pv. <i>savastanoi</i>		
NCPPB 3335	Wild-type strain isolated from olive	[3]
NCPPB 3335 Δ <i>hrpL::nptII</i>	<i>hrpL</i> mutant derived from NCPPB 3335 with Km resistance <i>nptII</i> cassette (Km ^R)	[4]
NCPPB 3335 Δ <i>hrpL</i>	<i>hrpL</i> mutant derived from NCPPB 3335 Δ <i>hrpL::nptII</i>	This work
<i>P. savastanoi</i> pv. <i>nerii</i>		
<i>Psn23</i> (ESC23)	Wild-type strain isolated from oleander	[5]
<i>Psn23</i> Δ <i>hrpL::nptII</i>	<i>hrpL</i> mutant derived from <i>Psn23</i> with Km resistance <i>nptII</i> cassette (Km ^R)	This work
<i>Psn23</i> Δ <i>hrpL</i>	<i>hrpL</i> mutant derived from <i>Psn23</i> Δ <i>hrpL::nptII</i>	This work
<i>P. savastanoi</i> pv. <i>mandevillae</i>		
Ph3 (CFBP 8832)	Wild-type strain isolated from dipladenia	[6]
Ph3 Δ <i>hrpL::nptII</i>	<i>hrpL</i> mutant derived from Ph3 with Km resistance <i>nptII</i> cassette (Km ^R)	This work
Ph3 Δ <i>hrpL</i>	<i>hrpL</i> mutant derived from Ph3 Δ <i>hrpL::nptII</i>	This work
<i>P. savastanoi</i> pv. <i>fraxini</i>		
NCPPB 1006	Wild-type strain isolated from ash	[7]
NCPPB 1006 Δ <i>hrpL::nptII</i>	<i>hrpL</i> mutant derived from NCPPB 1006 with Km resistance <i>nptII</i> cassette (Km ^R)	This work
NCPPB 1006 Δ <i>hrpL</i>	<i>hrpL</i> mutant derived from NCPPB 1006 Δ <i>hrpL::nptII</i>	This work
<i>P. savastanoi</i> pv. <i>retacarpa</i>		
CECT 4861	Wild-type strain isolated from broom	[8]
CECT 4861 Δ <i>hrpL::nptII</i>	<i>hrpL</i> mutant derived from CECT 4861 with Km resistance <i>nptII</i> cassette (Km ^R)	This work
CECT 4861 Δ <i>hrpL</i>	<i>hrpL</i> mutant derived from CECT 4861 Δ <i>hrpL::nptII</i>	This work
<i>P. syringae</i> pv. <i>tomato</i>		
DC3000	Wild-type strain isolated from tomato	[9]
<i>P. syringae</i> pv. <i>tabaci</i>		
CFBP 1621	Wild-type strain isolated from tobacco	[10]/W.S. Beach

Sp, spectinomycin; Cm, chloramphenicol; Km, kanamycin.

Table S2. Plasmids used in this study.

Name	Description	Reference
pGEM-T	Cloning vector containing ori f1 and <i>lacZ</i> (Ap ^R)	(Promega, USA)
pIAC4-Km	pGEM-T derivate, contains approximately 1.2 kb on each side of the <i>P. savastanoi</i> pv. <i>savastanoi</i> NCPPB 3335 <i>hrpL</i> gene (Ap ^R , Km ^R)	[4]
pFLP2	Contains a Flp recombinase (flipase) gene and <i>sacB</i> (Ap ^R)	[11]

Ap, ampicillin; Km, kanamycin.

Table S3. List and application of primers used in this work.

Purpose	Name	Sequence (5'→3')
<i>hrpL</i> probe ^a	<i>hrpL</i> F-29	CCTAGTGATCCTTGATGC
	<i>hrpL</i> R-381	CAAGCAATCAATGGCTGC
<i>nptII</i> probe	<i>nptII</i> F	GTGTAGGCTGGAGCTGCTTC
	<i>nptII</i> R	TTGCATCAGCCATGATGG
Checking for genomic cleanliness of the RNA	<i>catB</i> R1004	GCTCGACCAGAATGTCTTCG
	<i>catB</i> F516	CAAGCTGAAGATCGGTGCC
Real time quantitative PCR	<i>gyrA</i> F-180	GAGCGAGCTGGGTAACGACTGGAAC
	<i>gyrA</i> R-329	CAGCGTTGTGCCATCGACCGAA
	<i>hrpA</i> F-93	ATCAACAGCGTCAAGAGCAG
	<i>hrpA</i> R-267	ATTTGGTTGGAAAGGGCTTC
	<i>avrPto</i> F-295	GACAGGAGAGTCAGGAGTAAACC
	<i>avrPto</i> R-430	AGATCCGCTTGGGTTCATAG
	<i>hopAO2</i> -F	TGCGTTTGATGGTGACCGA
	<i>hopAO2</i> -R	ACCGCAATGGATATGTACCCG

^a These primers were also used to perform the verification of the possible mutant colonies obtained by PCR.

Table S4. Reads obtained in RNA-seq.

Sample	Raw reads	Clean reads ^a	Reads mapped to reference (%) ^b
Psv NCPPB 3335			
Replicate 1	41,289,538	33,083,850 (80.13%)	99.75
Replicate 2	40,704,762	33,925,884 (83.35%)	99.60
Total	81,994,300	67,009,734 (81.72%)	99.67
Psv NCPPB 3335 Δ<i>hrpL</i>			
Replicate 1	47,862,912	40,450,444 (84.51%)	99.67
Replicate 2	47,974,950	40,705,092 (84.85%)	99.65
Total	95,837,862	81,155,536 (84.68%)	99.66
Total RNA-seq	177,832,162	148,165,270 (83.32%)	99.67

^a Clean reads and percentage of accepted reads obtained after preprocessing with SeqTrimNext. ^b Percentage of clean reads that mapped with fully closed reference genome of *P. savastanoi* pv. *savastanoi* NCPPB 3335 including its native plasmids, pPsv48A, pPsv48B, and pPsv48C.

Table S5. Summary of Illumina RNA-seq data.

	Psv NCPPB 3335		Psv NCPPB 3335 Δ <i>hrpL</i>	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
Technology	Illumina	Illumina	Illumina	Illumina

Read length (nt)	75	75	75	75
Phred score (% \geq Q30)	>88%	>88%	>88%	>89%
Genome size (bp)	6,143,577	6,143,577	6,143,022	6,143,022
Sequencing depth ^a	344.2	351.6	420.8	423.5
CDS in chromosome	5597	5597	5596	5596
% CDS covered by reads	99.18	99.18	99.27	99.29
CDS in plasmids	172	172	172	172
% CDS covered by reads	100.00	99.42 ^b	100.00	100.00

^aSequencing depth was defined as number of nucleotides used for analysis divided by the genome size plus the three plasmids. ^bA CDS (coding sequence) from plasmid pPsv48C was not covered.

Table S6. Genes shared by in vitro and in silico analysis.

Accession number ^a	Protein ID ^a	Length ^b	Annotation	Gene
Type III secretion system (T3SS)				
PSA3335_RS10675	WP_002552422.1	368	YopN family type III secretion system gatekeeper subunit	<i>hrpJ</i>
PSA3335_RS10620	WP_004666089.1	359	EscU/YscU/HrcU family type III secretion system export apparatus	<i>hrcU</i>
			switch protein	
PSA3335_RS10600	WP_002552409.1	67	Hypothetical protein	<i>hrpT</i>
PSA3335_RS10590	WP_080393802.1	146	Type III secretion protein	<i>hrpG</i>
PSA3335_RS10585	WP_002552406.1	74	Type III secretion protein	<i>hrpF</i>
PSA3335_RS10560	WP_002552401.1	347	Type III secretion system helper protein	<i>hrpZ</i>
PSA3335_RS10555	WP_002552400.1	108	Membrane protein	<i>hrpA</i>
Helpers T3SS				
PSA3335_RS07245	WP_002554797.1	524	Two-domain harpin	<i>hopAK1</i>
PSA3335_RS15920	WP_004665222.1	96	Type III chaperone protein ShcV	<i>ShcV</i>
PSA3335_RS25240	WP_031597912.1	131	Type III chaperone protein ShcF	<i>ShcF</i>
T3SS effectors				
PSA3335_RS01620	WP_002551447.1	162	Hypothetical protein	<i>avrPto1</i>
PSA3335_RS25235	WP_002556083.1	223	Hypothetical protein	<i>avrRpm2</i>
PSA3335_RS02535	WP_002556043.1	257	Hypothetical protein	<i>hopAB1</i>
PSA3335_RS20790	Pseudogene	110	DUF1826 domain-containing protein	<i>hopAZ1</i>
PSA3335_RS02560	Pseudogene	1712	Type III effector protein XopAD	<i>xopAD</i>
Hypothetical proteins				
PSA3335_RS02555	WP_080719001.1	61	Hypothetical protein	<i>HP02555</i>
PSA3335_RS29775	Pseudogene	40	Hypothetical protein	<i>HP29775</i>
Signaling				
PSA3335_RS05360	WP_031599113.1	212	Hypothetical protein	<i>HP05360</i>
Secondary metabolism				
PSA3335_RS20310	WP_002552885.1	343	FAD: protein FMN transferase	<i>HP20310</i>

^a Accession number and protein ID in NCBI (<https://www.ncbi.nlm.nih.gov/>). ^bLength in amino acids.

Table S7. Structural genes of T3SS and genes associated with T3SS under regulation of HrpL in *P. savastanoi* pv. *savastanoi* NCPPB 3335.

Accession number ^a	Gene	O ^b	RNA-seq ^c			<i>hrp</i> value ^d	HrpL dependence in other pathovars ^e					
			<i>q</i> value	Log ₂ (fc)	Pto		Pph	Psy	Pla	Pja	Por	
T3SS pilus												
PSA3335_RS10685	<i>hrpK</i>	1	0.006	2.93	nd							
PSA3335_RS10680	<i>hrpL</i>	1	0.006	#	nd							
PSA3335_RS10675	<i>hrpJ</i>	1	0.006	-4.34	2779							
PSA3335_RS10670	<i>hrcV</i>	2	0.006	-2.81								
PSA3335_RS10665	<i>hrpQ</i>	3	0.006	-2.93								
PSA3335_RS10660	<i>hrcN</i>	4	0.006	-2.70								
PSA3335_RS10655	<i>hrpO</i>	5	0.006	-4.48								
PSA3335_RS10650	<i>hrpP</i>	1	0.006	-3.49		nd						
PSA3335_RS10645	<i>hrcQA</i>	2	0.006	-4.06		2585						
PSA3335_RS10640	<i>hrcQB</i>	3	0.006	-3.59								
PSA3335_RS10635	<i>hrcR</i>	4	0.006	-2.04								
PSA3335_RS10630	<i>hrcS</i>	5	NS	-4.15								
PSA3335_RS10625	<i>hrcT</i>	6	0.006	-1.71								
PSA3335_RS10620	<i>hrcU</i>	6	0.006	-1.92								
PSA3335_RS10605	<i>hrpV</i>	5	0.006	-1.18								
PSA3335_RS10600	<i>hrpT</i>	4	0.022	-2.34								
PSA3335_RS10595	<i>hrcC</i>	3	0.006	-2.40								
PSA3335_RS10590	<i>hrpG</i>	2	0.006	-3.67	2796							
PSA3335_RS10585	<i>hrpF</i>	1	0.006	-3.16	2796							
PSA3335_RS10580	<i>hrpE</i>	6	0.006	-1.73								
PSA3335_RS10575	<i>hrpD</i>	5	0.006	-1.32								
PSA3335_RS10570	<i>hrcJ</i>	4	0.006	-1.79								
PSA3335_RS10565	<i>hrpB</i>	3	0.006	-2.16								
PSA3335_RS10560	<i>hrpZ</i>	2	0.006	-2.60		2739						
PSA3335_RS10555	<i>hrpA</i>	1	0.006	-3.68		2739						
PSA3335_RS10550	<i>hrpS</i>	2	NS	0.03								
PSA3335_RS10545	<i>hrpR</i>	1	NS	0.00								

^a Accession number in NCBI (<https://www.ncbi.nlm.nih.gov/>). ^b O, operon. Number indicates order of gene within the operon (from 5' to 3'). Blue shading indicates first gene of operon and individually transcribed genes. ^c Genes identified by RNA-seq. Significantly differentially expressed genes have *q* values < 0.05; NS, not significant. Fold change (fc) refers to average expression rate obtained in $\Delta hrpL$ mutant relative to wild-type strain in two biological replicates. Negative (red) and positive (green) log₂ (fc) correspond to genes downregulated and upregulated, respectively, in $\Delta hrpL$ mutant relative to wild-type strain. #, indicates that the expression in the $\Delta hrpL$ mutant is zero. ^d Bioinformatic prediction of putative *hrp*-box upstream of start codon of genes encoded in *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome (*hrp* value \geq 2500); nd, not detected. ^e Red box, strains in which activation by HrpL of a homologous protein was identified by RNA-seq [12,13]. Pto, Pph, Psy, Pla, Pja, and Por: *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, *P. syringae* pv. *syringae* B728A, *P. syringae* pv. *lachrymans* 107, *P. syringae* pv. *japonica* MAFF 301072, and *P. syringae* pv. *oryzae* 1_6, respectively.

Table S8. T3Es genes under regulation of HrpL in *P. savastanoi* pv. *savastanoi* NCPPB 3335.

Accession number ^a	Gene	RNA-seq ^c		<i>hrp</i> value ^d	EffectiveDB ^e	HrpL dependence in other pathovars ^f					
		<i>q</i> value	Log ₂ (fc)			Pto	Pph	Psy	Pla	Pja	Por
Known T3Es											
PSA3335_RS10535*	<i>avrE1</i>		NS	nd	0.999						
PSA3335_RS01620	<i>avrPto1</i> ^b	0.006	-2.21	2832	1.000						
PSA3335_RS25235	<i>avrRpm2</i>	0.006	-0.77	2699	1.000						
PSA3335_RS05460	<i>hopA1</i>		NS	nd	nd						
PSA3335_RS10695	<i>hopA2</i>	0.006	1.28	nd	nd						
PSA3335_RS10490	<i>hopAA1</i>	0.006	-2.29	2728	nd						
PSA3335_RS02535	<i>hopAB1</i>	0.006	-0.87	2591	0.999						
PSA3335_RS24240	<i>hopAE1</i>	0.006	-1.16	nd	1.000						
PSA3335_RS29755*	<i>hopAF1-1</i>		NS	nd	1.000						
PSA3335_A0028	<i>hopAF1-1</i> ^b		NS	nd	1.000						
PSA3335_RS29760*	<i>hopAF1-2</i> ^b		NS	nd	nd						
PSA3335_RS14505	<i>hopAH2</i>		NS	nd	1.000						
PSA3335_RS24880	<i>hopAJ2</i>		NS	nd	nd						
PSPSV_B0010	<i>hopAO1</i> ^b	0.006	-0.59	nd	nd						
PSA3335_RS12020	<i>hopAO2</i> ^b		NS	2579	nd						
PSA3335_RS02305*	<i>hopAS1</i>		NS	nd	nd						
Not available	<i>hopAT1</i>		NS	nd	nd						
PSA3335_RS12045*	<i>hopAU1</i>	0.006	-0.84	nd	nd						
PSA3335_RS20790*	<i>hopAZ1</i>	0.006	-1.53	2770	nd						
PSA3335_RS19200	<i>hopBK1</i> ^b		NS	nd	nd						
PSA3335_RS02885*	<i>hopBL1</i> ^b		NS	nd	nd						
PSA3335_RS03825*	<i>hopBL2</i> ^b		NS	nd	nd						
PSA3335_RS02795	<i>hopBM1</i>		NS	2546	nd						
PSA3335_RS28230	<i>hopD1</i>		NS	nd	1.000						
PSA3335_RS21975	<i>hopG1</i>		NS	2634	1.000						
PSA3335_RS24700	<i>hopI1</i>	0.006	-1.02	nd	nd						
PSA3335_RS10525	<i>hopM1</i>	0.006	-1.29	nd	nd						
PSA3335_RS28225	<i>hopQ1</i>		NS	2554	0.999						
PSA3335_RS28250	<i>hopR1</i>	0.006	-0.60	nd	nd						
PSA3335_RS15915*	<i>hopV1</i>	0.006	-1.80	nd	nd						
PSA3335_RS29445	<i>hopW1</i>		NS	nd	nd						
New putativeT3Es											
PSA3335_RS02560	<i>xopAD</i>	0.032	-0.52	2789	1.000						

^a Accession number in NCBI (<https://www.ncbi.nlm.nih.gov/>). Asterisks indicate genes whose length in NCBI does not match which previously described in HopDatabase (<http://www.pseudomonas-syringae.org/>). ^b Genes whose HrpL dependency was previously demonstrated by RT-qPCR in *P. savastanoi* pv. *savastanoi* NCPPB 3335 [4,14,15]. ^c Genes identified by RNA-seq. Significantly differentially expressed genes have *q* values < 0.05; NS, not significant. Fold change (fc) refers to average expression rate obtained in

$\Delta hrpL$ mutant relative to wild-type strain in two biological replicates. Negative (red) and positive (green) \log_2 (fc) correspond to genes downregulated and upregulated, respectively, in $\Delta hrpL$ mutant relative to wild-type strain. ^d Bioinformatic prediction of putative *hrp*-box upstream of start codon of genes encoded in *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome (*hrp* value ≥ 2500); nd, not detected. ^e EffectiveDB [16] provides values (0.999–1) for proteins in which N-terminal T3SS targeting pattern is detected; nd, not detected. ^f Red box, strains in which activation by HrpL of a homologous protein was identified by RNA-seq [12,13]. Pto, Pph, Psy, Pla, Pja, and Por: *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, *P. syringae* pv. *syringae* B728A, *P. syringae* pv. *lachrymans* 107, *P. syringae* pv. *japonica* MAFF 301072, and *P. syringae* pv. *oryzae* 1_6, respectively.

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