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Variable Suites of Non-effector Genes Are Co-regulated in the Type III Secretion Virulence Regulon across the Pseudomonas syringae Phylogeny

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Abstract

Pseudomonas syringae is a phylogenetically diverse species of Gram-negative bacterial plant pathogens responsible for crop diseases around the world. The HrpL sigma factor drives expression of the major P. syringae virulence regulon. HrpL controls expression of the genes encoding the structural and functional components of the type III secretion system (T3SS) and the type three secreted effector proteins (T3E) that are collectively essential for virulence. HrpL also regulates expression of an under-explored suite of non-type III effector genes (non-T3E), including toxin production systems and operons not previously associated with virulence. We implemented and refined genome-wide transcriptional analysis methods using cDNA-derived high-throughput sequencing (RNA-seq) data to characterize the HrpL regulon from six isolates of P. syringae spanning the diversity of the species. Our transcriptomes, mapped onto both complete and draft genomes, significantly extend earlier studies. We confirmed HrpL-regulation for a majority of previously defined T3E genes in these six strains. We identified two new T3E families from P. syringae pv. oryzae 1_6, a strain within the relatively unexplored phylogenetic Multi-Locus Sequence Typing (MLST) group IV. The HrpL regulons varied among strains in gene number and content across both their T3E and non-T3E gene suites. Strains within MLST group II consistently express the lowest number of HrpL-regulated genes. We identified events leading to recruitment into, and loss from, the HrpL regulon. These included gene gain and loss, and loss of HrpL regulation caused by group-specific cis element mutations in otherwise conserved genes. Novel non-T3E HrpL-regulated genes include an operon that we show is required for full virulence of P. syringae pv. phaseolicola 1448A on French bean. We highlight the power of integrating genomic, transcriptomic, and phylogenetic information to drive concise functional experimentation and to derive better insight into the evolution of virulence across an evolutionarily diverse pathogen species.

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Introduction

Many Gram-negative bacteria attach to host cells and translocate effector proteins into them via type III secretion systems (T3SS). Such systems are necessary for pathogenesis, are horizontally transferred across species, and are accompanied by dynamically evolving repertoires of type III effector (T3Es) genes [1,2]. The T3SS is essential for Pseudomonas syringae pathogens to thrive in plant tissues. P. syringae represents an excellent example of the plasticity of T3E repertoires [3]. Despite a collectively broad host range for the species, individual isolates of P. syringae typically display pathogenic potential on a limited set of plants and either elicit immune responses, or simply fail to thrive on other plant species. Strains can be isolated from diseased plants, as epiphytes from healthy plants [4], and from various environmental sources [5].
Pseudomonas syringae are environmentally ubiquitous bacteria of wide phylogenetic distribution, which can cause disease on a broad range of plant species. Pathogenicity requires the master regulator HrpL. HrpL controls the activation of virulence factor genes, including those encoding the type III secretion system which facilitates translocation of bacterial proteins into host cells. Here we overlaid transcriptome profiling of genes onto their phylogenetic distribution by characterizing the HrpL regulon across six diverse strains of P. syringae. We identified novel putative virulence factors, discovered two novel effector families, and functionally characterized an operon most likely involved in secondary metabolism that we show is required for virulence. We demonstrated that the size and composition of the HrpL regulon varies among strains, and explored how genes are recruited into, or lost from, the virulence regulon. Overall, our work widens the understanding of P. syringae pathogenicity and presents an experimental paradigm extensible to other pathogenic bacterial species.

The hrp/hrc group I T3SS is essential for P. syringae pathogens to cause disease on plants [1,6]. The genes that encode the hrp/hrc T3SS and accessory proteins are clustered in a conserved pathogenicity island in P. syringae [7]. The genes for the associated T3E can be scattered across the genome, often in association with mobile elements indicative of horizontal transmission [8–10]. Each strain’s T3E repertoire ranges from 15–30 genes sampled from at least 57 different families and these collectively modify host cell biology to suppress immune response and favor bacterial proliferation and dispersion. However, the action of individual T3E proteins can be recognized by plant host disease resistance proteins, and this triggers immune responses sufficient to limit pathogen growth [11]. The conflicting selective pressures to retain a collection of T3E sufficient to suppress host defenses without triggering effector-specific immune responses [11] drives diversity in the suites of T3Es in plant pathogenic P. syringae isolates [3].

Transition from saprophytic to epiphytic or pathogenic lifestyle requires significant transcriptional reprogramming. Expression of genes encoding the P. syringae T3SS structural components and the associated T3E suite is controlled by the ECF-type sigma factor HrpL [12–14]. The expression of hrpL is induced in bacteria that encounter the leaf environment [13]. Subsequently, HrpL binds to promoters carrying a “hrp-box” consensus sequence to up-regulate the expression of the corresponding gene(s) [12–15].

Previous studies in P. syringae identified proteins that are neither T3E nor structural components of the T3SS (hereafter, non-T3E), but are HrpL-regulated [3,16–19]. Non-T3E proteins are coordinately regulated with the T3SS and its substrates were also found in other T3SS-expressing plant pathogens such as Escaexual aimholeora [20], Ralstonia solanacearum [21], Xanthomonas campestris pv. vesicatoria [22,23] and Pectobacterium carotovora [24]. Some HrpL-regulated non-T3E genes affect virulence on host plants in the well-studied strain P. syringae pv. tomato DC3000 (PstDC3000); these include the corR regulator of coronatine toxin production [18,25]. Notably, CorR expression is not HrpL-regulated in other strains, such as P. syringae pv. glycinea PG1180 [26].

Multi-Locus Sequence Typing (MLST) separates plant pathogenic P. syringae into at least 5 distinct phylogenetic groups [3,27]. The fifth group, represented originally by P. syringae pv. maculicola ES4326, was recently renamed P. cannabina pv. alismae ES4326 [28]. Many P. syringae genome sequences are now available, including three closed genomes from isolates representing major pathogen clades [29–31], and ~120 additional draft sequences. Newly sequenced genomes also trace P. syringae disease outbreaks across the globe and over time [3,32–39] attesting to the continued importance of the species. Recently, isolation and sequencing of saprophytic and epiphytic strains provided insight into a subgroup from group II that carries a non-canonical T3SS [40]. To date, transcriptome analyses using high throughput short read cDNA sequencing (RNA-seq) have been applied only to PstDC3000, providing a well-curated reference gene annotation, but not specifically informing studies of the HrpL regulon [41–44].

In this study, we defined the HrpL regulon of six distinct strains of P. syringae with complete or draft genomes using RNA-seq coupled with the GENE-counter software package [45–47]. We sought primarily to compare the diversity of non-T3E HrpL-regulated genes between strains and secondarily to determine if there were additional type III effectors not found in our DNA-based analyses [3]. We detect non-T3E genes regulated directly or indirectly by HrpL. Those directly regulated by HrpL are distributed throughout the P. syringae clades in a mosaic pattern. However, most are either absent or not HrpL-regulated in MLST group II. We demonstrate that a novel cluster of non-T3E genes is required for P. syringae pv. phaseolicola 1448A virulence. We also identified two novel T3E families from a previously understudied clade. Our study reveals the mechanisms for gene recruitment into, and loss from, the key virulence regulon in P. syringae, and provides a roadmap for future functional studies.

Results

The HrpL regulons of six phylogenetically diverse P. syringae isolates are defined by RNA-seq

We defined the HrpL regulons of P. syringae pv..phaseolicola strain 1448A (Pph1448A), P. syringae pv. lachrymans strain 107 (Pll107) representing MLST group III; P. syringae pv. syringae strain B728a (PssB728a), P. syringae pv. japonica strain MAFF 300172 PT (Pja) representing MLST group II; P. syringae pv. tomato strain DC3000 (PstDC3000) representing MLST group I and P. syringae pv. oryzae strain 1_6 (Por), belonging to the relatively poorly studied clade, MLST group IV [3,27]. The native hrpL gene from each isolate was cloned downstream of an arabinose-inducible promoter for controlled, high-level expression in the strain of origin. Isogenic strains carrying either the appropriate hrpL construct, or an empty vector (EV) as negative control, were grown with arabinose to induce the expression of the cloned hrpL gene in a minimal medium [19]. Expression of the native hrpL was repressed by addition of peptone to the media [48]. Figure S1 depicts our experimental pipeline and control validation.

We generated Illumina cDNA libraries from two biological replicates of each strain. Because our goal was to compare transcript abundance more than to improve annotation of transcribed genes, we used a simple cDNA method to minimize the RNA processing steps where transcripts could be lost. Therefore, we did not enrich for 5’ ends or distinguish transcript orientation. Transcript abundance was compared between isogenic HrpL and EV samples using GENE-counter [45]. Similar to other RNA-seq analysis methods like EdgeR or DESeq [49,50], GENE-counter determines differential expression. While EdgeR and DESeq use the standard negative binomial distribution, GENE-counter relies on the negative binomial p distribution which better accounts for the over-dispersion observed in mRNA-seq data [51–53]. We bootstrapped the GENE-counter output for each isolate (Materials and Methods) to control for noise introduced by sample normalization. Between 1.6 and 5.6 million
unambiguous reads per sample (mapping to only one location in the reference genome) were used for our analyses (Table 1). The sequencing depth ranged from 9.5 to 16.2 times the genome size, with the exception of the P\text{h}\text{i}_{1448A} samples, which we sequenced to higher coverage (Table 1). On average 93.5% of the total number of annotated coding genes in a genome were covered by at least one read in at least one sample (Table 1). Bootstrapped-GENE-counter analysis established a median read count for every sample, with one read in at least one sample (Table 1). Bootstrapped-GENE-counter analysis has been used widely to infer transcript abundance from RNA-seq data 

We analyzed the same P\text{h}p\text{DC3000} RNA-seq data set using either the complete P\text{h}p\text{DC3000} genome sequence [30] or a draft P\text{h}p\text{DC3000} genome sequence [36] as references. The Pto\text{DC3000} genome sequence covers 85% of genes at over 90% of their length [36]. Using either the complete or the draft genome as a reference resulted in similar sequencing depths (Table 1). Using the draft genome as a reference, GENE-counter identified 124 HrpL-upregulated genes out of the 133 found using the complete P\text{h}p\text{DC3000} genome (Table 2). Most of the genes that were not identified as differentially expressed using the draft genome were missing from the draft genome (data not shown). The high correlation between the Log(\text{median} q\text{-value}) of genes in the two data sets (Figure 1C) indicates that our method will effectively identify the majority of genes of the HrpL regulon from P. syringae isolates for which only a high quality draft genome is available.

RNA-seq successfully captures the HrpL regulon for P\text{h}p\text{DC3000} and P\text{h}i\text{1448A}

To further validate our pipeline to define HrpL-regulated genes, we compared our manually curated list of 110 P\text{h}p\text{DC3000} HrpL-regulated genes (Table 2) to HrpL-regulated genes identified by three previous studies: one promoter probe study using an arabinose-inducible h\text{rp}L gene and two custom microarray analyses which compared expression between wild type and h\text{rp}L deletion mutant strains [16,17,19]. These studies produced largely overlapping, but not identical, lists of putatively HrpL-regulated genes (Table S3). Our P\text{h}p\text{DC3000} HrpL-regulated gene set included 57
out of the 66 genes previously identified as HrpL-regulated in at least two of the previous studies (Table S5), even though our induction and analysis methods differed from these studies. 96 of the 110 genes we identified were also found to be HrpL-regulated in at least one of the previous studies [16,17,19] or were downstream genes in HrpL-regulated operons (Table 2). Overall, we found 91% of the previously identified HrpL-regulated genes in _P. syringae_. Our analysis also identified 14 novel HrpL-regulated genes (Table 2); six out of eight tested were confirmed to be HrpL-regulated using qRT-PCR (Table 3, see below).

Notably, four of the nine missing genes were not present in our laboratory strain, which has lost part of the _P. syringae_ plasmid A. One gene, _shca_ (_PSPTO_3535) was found differentially expressed in our analysis but had a B-value less than 50%. Further, GENE-counter discards RNA-seq reads that map non-uniquely to more than one location in the genome, and HrpL-regulated duplicated genes account for three missing _P. syringae_ genes: T3E genes _hopAM1-1_ (_PSPTO_1022) and _hopQ1-2_ (_PSPTO_4732), and the non-T3E gene _plcA2_ (_PSPTO_B0005_) (Table S5). Finally, _hopK1_ (_PSPTO_0044), was covered by RNA-seq reads but the differences in expression in HrpL and EV treatments were not statistically significant (Table S1, S5).

Two previous studies focused on the identification of HrpL-regulated genes in _P. syringae_ [18,19] and identified 43 HrpL-regulated genes comparing expression between wild type and _hopL_ mutants. We identified 35 (~80%). Four of the missing eight genes were covered by reads but not found significantly differentially expressed, _hopAK1_ (_PSPHL_1424), a gene encoding a MarR transcriptional regulator (_PSPHL_1519), _awRps4_ (_PSPHL_A0067_), and _hopAS1_ (_PSPHL_4736_). Those four genes had a median read coverage ranging from 100 to 1000, indicating that the absence of differential expression in our analysis is not due to weak or undetectable levels of expression. One, _PSPHL_2294 is a pseudogene. _PSPHL_1525 encoding a putative effector related to _Ralstonia_ Hpx30 [55], _PSPHL_A0009_ and _A00075_ encoding truncated _hopW1_ are duplicated and had very low to no read coverage (Table S5). Our GENE-counter analysis pipeline results are consistent with previous transcriptional studies, reinforcing the validity of our methods. Additionally, we identified robustly HrpL-induced genes that were not previously identified.

**Quantitative RT-PCR analyses predominantly confirm our RNA-seq data**

We identified between six and 32 genes previously not known to be HrpL-regulated in each strain with corresponding q-values ranging from E-02 to E-54 (Table 2, Table S3). Some of these are shared across strains. We could not identify a consensus upstream _hrp_ box in the promoters of several, and suggest that these could be indirectly activated by HrpL. We performed qRT-PCR using samples derived from strains expressing HrpL in the _pBAD_ system and confirmed 19 of 23 tested (Figure S2). Additionally, we confirmed HrpL-dependent expression of 19 genes out of 20 tested, by comparing wild type expression with expression in a _hopL_ deletion mutant in _hrpL_-inducing minimal medium (Table 3, Figure S3). We observed a high correlation between RNA-seq data and either qRT-PCR profiling method, especially for genes with a q-value > E-03 (Table 3, Figures S2, S3). In sum, we identified the majority of previously identified HrpL-regulated genes in two well-studied strains and we confirmed wild type HrpL regulation for nearly all of the newly identified members of this key virulence regulon.

**RNA-seq identifies new T3E genes**

Most of the known T3E and candidate T3E genes in our tested strains and those previously defined by similarity and/or functional criteria were included in the HrpL regulons we defined in our RNA-seq analyses (Figure S4). Most of strains used in this study had previously been screened for novel type III effector genes by functional translocation assays with the exception of _Por_ and _Pja_ [3,19]. Therefore, we searched the _Por_ and _Pja_ HrpL regulons for potential novel effector genes based on the criteria of having an identifiable upstream _hrp_ box sequence and no homology to previously identified T3E families. We chose six _Por_ genes (Por_0547_07284, 04644, 04640, 03530, 02145, and 04371) to
investigate as potentially encoding novel T3Es. Pja also carries a gene homologous to Porcurated_04644; but only the Por allele was tested. All six putative T3E were tested for their ability to be translocated via a native T3SS using an established assay [56] (Materials and Methods) from Pto DC3000D28E, an “effector-less” Pto DC3000 strain [57]. Only Pto DC3000D28E carrying Por curated_02784-D79avrRpt2 or Por curated_04640-D79avrRpt2 triggered a Hypersensitive Response (HR) in Col-0 (Figure 2A). We verified that HA-tagged versions of all six T3E candidates were expressed in Pto DC3000D28E indicating that lack of HR in our translocation assay was unlikely due to a lack of protein accumulation (Figure 2B). No HR was observed in the rps2 mutant, indicating that the response was avrRpt2-specific and not the result of toxicity.

These two new P. syringae effectors will henceforth be referred to as HopBH1 Por and HopBI1 Por according to proposed T3E naming guidelines [58]. None of the 19 P. syringae strains for which we previously performed comparative genomic analysis encode either hopBH1 or hopBI1 [3]. However, each can be found in P. syringae strains isolated from various sources ranging from non-symptomatic plants to snow [33,35,40,59–62] (Figure S5). Amino acid sequence alignments suggest that HopBH1 is a bi-modular effector exhibiting sequence conservation within its C-terminal domain and sequence diversity toward its N-terminal half (Figure S6). In the non-pathogenic strain Psy642, the putative HopBH1 protein appears to have been disrupted by a frameshift mutation, leading to two putative open reading frames designated as ORF29-30 [40]. Phylogenetic analysis of strains carrying either hopBH1 and/or hopBI1 indicates that both effector genes occur with a mosaic distribution across the P. syringae, but are absent from the phylogenetic group III [3,27] (Figure S5). Neither HopBH1 nor HopBI1 contain known protein folds, nor do they display sequence or structural homology to proteins of known function.

The HrpL regulons are diverse across isolates

The composition of the HrpL regulon across strains was surveyed by functional classification based on protein annotation and sequence homology determined by BLASTP (Table S6). As summarized in Figure 3 and Table S7, PtoDC3000 and Por possess the largest and most diverse HrpL regulons among the sampled strains, while the Group II strains Pja and PSYB728a have the smallest. We are confident that the less complex HrpL regulons are not a sampling artifact, because the data collected from Pja has a transcriptome depth similar to the other strains, and the PSYB728a
Table 3. Real time RT-PCR analyses predominantly confirm RNA-seq data.

| Genes tested | Annotation | Median Q-value (Genecounter) | Putative hrp-box | pBAD system*# | Fold induction in pBAD system | Native system* | % expression in ΔhrpL vs. WT |
|--------------|------------|------------------------------|------------------|---------------|-------------------------------|---------------|-----------------------------|
| PSPTO_4332   | hypothetical protein | 4.66E-13                     | −                | +             | 15.3                         | +             | 3.8                         |
| PSPTO_2130   | LuxR family DNA-binding response regulator | 3.45E-10                     | +                | +             | 5.2                          | +             | 19.0                        |
| PSPTO_3086   | transcriptional regulator | 3.75E-10                     | −                | +             | 24.0                         | +             | 4.1                         |
| PSPTO_2129   | sensory box histidine kinase/response regulator | 4.86E-05                     | −                | +             | 3.4                          | +             | 21.3                        |
| PSPTO_2208   | htpG heat shock protein 90 | 9.82E-05                     | −                | +             | 2.7                          | +             | 53.6                        |
| PSPTO_0671   | macrolide efflux protein | 1.51E-04                     | +                | +             | 2.8                          | ND            | ND                          |
| PSPTO_1843   | aspartate kinase | 1.48E-03                     | −                | −             | 0.9                          | ND            | ND                          |
| PSPTO_4716   | hypothetical protein | 1.89E-02                     | −                | −             | 1.5                          | ND            | ND                          |
| PSPPH_A0112  | phosphoglycerate mutase family protein | 3.11E-11                     | −                | +             | 4.8                          | +             | 20.5                        |
| PSPPH_A0110  | hypothetical protein | 3.94E-10                     | −                | ND            | ND                           | +             | 53.6                        |
| PSPPH_A0109  | sulfotransferase, putative | 7.96E-08                     | −                | +             | 4.3                          | +             | 51.6                        |
| PSPPH_1906   | LuxR family DNA-binding response regulator | 5.51E-07                     | +                | +             | 4.9                          | +             | 4.1                         |
| PSPPH_0762   | hypothetical protein | 4.45E-03                     | −                | +             | 4.4                          | +             | 9.9                         |
| PSPPH_A0106  | hypothetical protein | 7.49E-03                     | −                | ND            | ND                           | +             | 46.4                        |
| PSPPH_A0108  | adenosylmethionine-8-amino-7-oxononanoate aminotransferase | 1.27E-02                     | −                | ND            | ND                           | +             | 36.7                        |
| Psyr_0737    | putative transmembrane protein | 1.00E-46                     | +                | +             | 23.3                         | +             | 8.0                         |
| Psyr_0027    | hypothetical protein | 1.64E-14                     | −                | +             | 4.3                          | −             | 84.5                        |
| PORcurated_00518 | hypothetical protein | 2.49E-54                     | +                | +             | 84.3                         | +             | 2.0                         |
| PORcurated_04644 | Methyltransferase small domain | 1.16E-37                     | +                | +             | 48.4                         | +             | 3.5                         |
| PORcurated_03530 | hypothetical protein | 3.03E-30                     | +                | +             | 19.5                         | +             | 34.2                        |
| PORcurated_04640 | hypothetical protein | 1.72E-27                     | +                | +             | 19.8                         | +             | 42.3                        |
| PORcurated_04022 | Alkylated DNA repair protein | 4.24E-24                     | −                | +/−           | 1.7                          | ND            | ND                          |
| PORcurated_04648 | hypothetical protein | 2.35E-14                     | −                | +             | 4.1                          | +             | 41.7                        |
| PORcurated_04371 | hypothetical protein | 3.39E-24                     | +                | +             | 5.2                          | +             | 30.2                        |
| PORcurated_04025 | hypothetical protein | 1.99E-11                     | −                | +             | 3.4                          | ND            | ND                          |
| PORcurated_04024 | Domain of unknown function (DUF1883) | 1.06E-04                     | −                | 0.6           | ND                           | ND            | ND                          |

*+ found up-regulated by qRT-PCR; − no up-regulation. ND, not determined. See Figure S2 and S3 for detailed qRT-PCR results.

# expression compared between Ps (pBAD::EV) strain and Ps (pBAD::hrpL) strain grown in media containing arabinose.

expression compared between a wild type strain and an isogenic clean hrpL deletion mutant grown in MM media.
doi:10.1371/journal.ppat.1003807.t003
HrpL regulon was sampled at relatively high depth compared to our other transcriptomes. We conclude that HrpL regulons vary in size and composition across the *P. syringae* phylogeny.

Recruitment of genes into and out of the HrpL regulon

We observed variable HrpL-dependent expression for several highly conserved non-T3E genes present in all six strains (Table S6). We identified polymorphisms in the *hrp*-box sequences from two of these (Figure 4A). In the first case, new HrpL-regulated genes we identified, PSPTO_2130, PSPPH_1906, and Lac107_00061530, are orthologs that encode a DNA-binding response regulator. HrpL-dependent induction was confirmed by qRT-PCR (Table 3, Figure 4B). Orthologous genes are also present in *Pja*, *Psy* B728a, *Por* (Pjap_00016990, Psyr_1940, and Porcurated_00527, respectively) but were not identified as differentially expressed (Table S1). PSPTO_2130 and all of its orthologs have conserved *hrp*-box motifs. However, the promoters of the orthologs from *Por* and all other group II strains contain single nucleotide polymorphisms (in red, Figure 4A) in the consensus *hrp*-box sequence. Our RNA-seq data suggested that expression of these polymorphic alleles was not HrpL-dependent, a finding confirmed by qRT-PCR performed with both of our HrpL-regulation experimental tests (Figure 4B, Figure S7A).

PSPTO_2130 and its orthologs are part of a putative operon composed of four genes, PSSTO_2130, PSPPH_1906, and Lat107_00061530, are orthologs that encode a DNA-binding response regulator. HrpL-dependent induction was confirmed by qRT-PCR (Table 3, Figure 4B). Orthologous genes are also present in *Pja*, *Psy* B728a, *Por* (Pjap_00016990, Psyr_1940, and Porcurated_00527, respectively) but were not identified as differentially expressed (Table S1). PSPTO_2130 and all of its orthologs have conserved *hrp*-box motifs. However, the promoters of the orthologs from *Por* and all other group II strains contain single nucleotide polymorphisms (in red, Figure 4A) in the consensus *hrp*-box sequence. Our RNA-seq data suggested that expression of these polymorphic alleles was not HrpL-dependent, a finding confirmed by qRT-PCR performed with both of our HrpL-regulation experimental tests (Figure 4B, Figure S7A).

Unusually, the *hrp*-box sequences were located within the first ORF of the putative operons of PSPTO_2130 and its orthologs. We monitored HrpL-dependent expression using qRT-PCR of all genes from PSPTO_2130 to 2128 from three strains (Figure S8B, C, D). In none of these strains was the first gene of the operon, containing the putative *hrp*-box, differentially expressed. By contrast, HrpL-dependent expression was observed for genes downstream of the predicted *hrp*-box, including coding sequences, PSPTO_2130 and PSPPH_1906, in all but the group II reference
strain \textit{PstB728a} (Figure S8). Deletion mutants in \textit{PstDC3000} and \textit{Pph1448A} of \textit{PSPTO_2130} and \textit{PSPPH_1906} did not display any growth defect on Arabidopsis accession Col-0 or French bean cultivar Tendergreen (susceptible to \textit{PstDC3000} and \textit{Pph1448A}, respectively) (data not shown). Thus, the role of \textit{PSPTO_2130} and its orthologs in virulence remains unclear.

In the second case, \textit{PSPTO_2105} and its orthologs, which encode a putative ApbE-family protein, are highly conserved across \textit{P. syringae} and are HrpL-regulated in \textit{Pph1448A}, \textit{Pla107}, \textit{PstDC3000} and \textit{Pst} but not in the group II strains \textit{PstB728a} or \textit{Pja} (Table S5, S6). qRT-PCR (Figure 4C, Figure S7B) support our RNA-seq data. \textit{PSPTO_2105} is required for full virulence of \textit{PstDC3000} on Arabidopsis [18]. We also observed significantly reduced virulence when we tested two independent deletion mutants of the \textit{Pph1448A} ortholog \textit{PSPPH_1855} for growth on the native host, French beans (Figure S9). Every group II strain analyzed has variations in the otherwise well conserved \textit{hrp}-box sequence in at least two positions (Figure 4A). Collectively, these data demonstrate that promoter erosion within the \textit{hrp}-box is a mechanism to remove genes from the virulence regulon.

**HrpL regulons of isolates from phylogenetic group II contain fewer non-T3E genes**

Both \textit{PstB728a} and \textit{Pja} appear to have relatively small HrpL regulons; both belong to the MLST group II. To address whether this was a general feature of group II strains, and to address the distribution of the genes that we identified experimentally across the phylogeny, we extended our investigation of non-T3E HrpL regulon diversity to BLAST homology searches of 44 sequenced \textit{Pseudomonas} spp. strains [3,35,63]. Our non-T3E gene search set included genes we experimentally confirmed for HrpL-dependent expression, genes that encoded proteins found not to be translocated, or genes unlikely to encode a translocated product by annotation. We removed T3SS genes and known T3Es (Figure 5). Most of the directly HrpL-regulated non-T3E genes we identified are absent from group II strains, but distributed across strains from groups I and III. Some are present in the previously described group IV and V, as well as the novel MLST groups VII, IX, X (Berge et al., personal communication, Figure S5) for which we had limited sampling. Further, the promoters of group II homologs of \textit{PstDC3000} are divergent, and lack canonical \textit{hrp}-boxes (data not shown). Thus, not only do group II strains possess fewer known T3E genes on average than the other phylogroups, group II strains also possess fewer non-T3E genes in their HrpL regulon suggesting a potential shift in virulence mechanisms of this clade [3].

**Recruitment of a novel gene cluster into an \textit{avrD}-containing virulence operon in \textit{Pph1448A}**

Both \textit{Pph1448A} and \textit{Pla107} contain \textit{avrD}, a gene required for synthesis of syringolides, small molecules sufficient for HR on soybean cultivars expressing the \textit{Rpg4} disease resistance gene [65–67]. \textit{avrD} is a non-T3E gene, as defined above (Figure 5), and its expression in \textit{E. coli} is sufficient for production of syringolides [65]. RNA-seq analysis identified a series of orthologous, HrpL-regulated genes directly downstream of \textit{avrD} in both \textit{Pph1448A} and \textit{Pla107} (Table S3, S6). In \textit{Pph1448A}, those genes are arranged in two clusters composed of \textit{PSPPH_A0112-A0110} and \textit{PSPPH_A0109-A0106}, which are flanked by transposable elements (Figure 6A). While most of these genes seem to encode hypothetical proteins, \textit{PSPPH_A0112}, \textit{A0109}, \textit{A0108}, \textit{A0107} encode putative enzymes: a phosphoglycerate mutase, a sulfo-
Figure 4. *hrp*-box mutations associated with differential HrpL-dependent up-regulation of *PSPTO_2105* and *2130* orthologs. (A) *PSPTO_2105* and *PSPTO_2130* *hrp*-box sequence variation across *P. syringae* strains as presented in [3]. In blue, canonical *hrp*-box nucleotides. In red, nucleotides of divergent *hrp*-box sequences. (B) HrpL-dependent expression of *PSPTO_2130* orthologs across *P. syringae* strains. qRT-PCR analysis was performed on RNA samples derived from isogenic strains expressing, or lacking, an arabinose-inducible *hrpL* gene. Expression was normalized to *gap*1. Relative expression: each EV sample was set to 1 and HrpL samples normalized to the corresponding EV samples. Error bars represent SD. (C) HrpL-dependent expression of *PSPTO_2105* orthologs across *P. syringae* strains, as above. Each experiment was repeated twice.

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transferase, an amino transferase, and an oxidoreductase respectively. We confirmed the HrpL-dependent expression of PSPPH_A0112, A0110, A0109, and A0107 (Table 3, Figure S2, and S3). This operon is typically found as a presence/absence polymorphism; when present, it is almost always downstream from avrD (Figure 6B). PSPPH_A0111 corresponds to a 99 bp sequence present in Pto DC3000, P. syringae pv. morsii (Pmo), P. syringae pv. glycinea R4 (PgyR4), P. syringae pv. tomato T1 (PtoT1), and P. syringae pv. actinidiae (Pan) but not annotated as an ORF, due to incomplete sequencing of the upstream region. Yellow boxes indicate absence of genes based on homology searches. The light blue boxes indicate the presence of a divergent ORF with upstream hrp-box sequence present. S, indicates that the gene is present as a single gene. O, indicates that the gene is present in an operon. + or − indicate that the gene was differentially expressed or not in our RNA-seq data. ND indicates not determined: PSPTO_A00030 is absent from our PtoOc3000 laboratory strain, and a homologous sequence of Psyr_0737 is present in Pja genome but was not annotated as an ORF.

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Figure 5. Strains of phylogenetic group II have the fewest non-effector genes in their HrpL regulons. “Non-effector” genes are listed across the top; Pseudomonas genomes, color-coded by phylogenetic group, on the left, according to Figure S5 (Pseudomonas UB246 was not included, because it belongs to a divergent pseudomonad lineage). Only the first gene of an operon is represented. Dark blue box indicates presence of full-length ORFs (with at least 80% nucleotide identity and 40% coverage), by similarity search as well as the presence of a hrp-box in the 500 bp upstream region. Grey boxes indicate that the corresponding gene was present, but the presence of putative hrp-boxes could not be determined, due to incomplete sequencing of the upstream region. Yellow boxes indicate absence of genes but present but either no hrp-boxes were detected in the upstream region or hrp-boxes are presumably not functional (because divergent from the hrp-box for which HrpL-dependent expression was confirmed). White boxes indicate absence of genes based on homology searches. The light blue boxes indicate the presence of a divergent ORF with upstream hrp-box sequence present. S, indicates that the gene is present as a single gene. O, indicates that the gene is present in an operon. + or − indicate that the gene was differentially expressed or not in our RNA-seq data. ND indicates not determined: PSPTO_A00030 is absent from our PtoOc3000 laboratory strain, and a homologous sequence of Psyr_0737 is present in Pja genome but was not annotated as an ORF.

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We confirmed the HrpL-dependent expression of PSPPH_A0112, A0110, A0109, and A0107 (Table 3, Figure S2, and S3). This operon is typically found as a presence/absence polymorphism; when present, it is almost always downstream from avrD (Figure 6B). PSPPH_A0111 corresponds to a 99 bp sequence present in Pto DC3000, P. syringae pv. morsii (Pmo), P. syringae pv. glycinea R4 (PgyR4), P. syringae pv. tomato T1 (PtoT1), and P. syringae pv. actinidiae (Pan) but not annotated as an ORF, due to incomplete sequencing of the upstream region. Yellow boxes indicate absence of genes based on homology searches. The light blue boxes indicate the presence of a divergent ORF with upstream hrp-box sequence present. S, indicates that the gene is present as a single gene. O, indicates that the gene is present in an operon. + or − indicate that the gene was differentially expressed or not in our RNA-seq data. ND indicates not determined: PSPTO_A00030 is absent from our PtoOc3000 laboratory strain, and a homologous sequence of Psyr_0737 is present in Pja genome but was not annotated as an ORF.

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We generated two independent deletion mutants for avrD and PSPPH_A0107 (ΔavrD #1 and 2, ΔPSPPH_A0107 # 1 and 2, respectively) and tested their growth on French bean cv. Tendergreen (Figure 6D). All mutants displayed reduced growth compared to wild type Pph1448 but either very weakly or not at all in the ΔhrpL mutant (Figure 6C). We confirmed that the HrpL-dependent expression of several downstream genes was not disrupted by mutations (Figure S10). However, PSPPH_A0112, A0107 and A0106 were consistently slightly up-regulated in avrD mutants compared to the wild type. The intact remaining hrp-box is closer to PSPPH_A0112-
Identification of two novel HrpL-regulated type III effectors in Por

We identified HopBH1\textsubscript{por} and HopB1\textsubscript{por}, defining two novel effector families. Both have a mosaic phylogenetic distribution across \textit{P. syringae} [35,40,63] (and an unpublished strain, TLP2, JGI taxon ID: 2507262033). Both are present in CC1513 and CC1629, two other strains belonging to the MLST group IV. They appear to be absent from sequenced MLST group III strains. HopBH1 has a bi-modal structure. The \(~170\) amino-acid N-terminus is divergent compared to the relatively well conserved \(~250\) amino acid C-terminal domain across HopBH1 alleles (Figure S6). The HopBH1 C-terminal domain is \(50\)% identical to a protein from \textit{P. fluorescens} SS101 which lacks a putative \textit{hp-box} or a T3SS secretion competent N-terminal sequence [68], suggesting that it may have been recruited as an effector by N-terminal assortment [69]. Several putative proteins present in \textit{Pantoea}, \textit{Serratia}, \textit{Burkholderia} species, as well as \textit{Myxobacteria}, display \(\approx 50\)% identity with the HopBH1 C-terminal domain. Remarkably, about \(150\) amino acids of the HopBH1 C-terminal domain also shares \(40\)% identity with...
part of the ~1000 amino acid long P. savastanoi pv. savastanoi NCPPB3335 HrpK. Notably, this hrpK gene (PSPTO_3335-2516) is from a rhizobia-like type III secretion and is different from the hrpK gene (PsST2486-1509) of canonical T3SS conserved in plant pathogenic P. syringae [70]. HopBH appears to be confined to Pseudomonas. Neither HopBH1, nor HopBH1 display similarity to known effectors. Their virulence functions remain to be determined.

HrpL regulons include diverse non-T3E genes some of which are known virulence factors

Although analysis of type III virulence systems focuses mainly on the characterization and function of T3SS and T3E proteins, several non-T3E genes are co-regulated with the T3SS. They encode hypothetical proteins, transporters, or enzymes likely involved in secondary metabolism (Figure 5). In contrast to T3E genes, for which functional redundancy is predominant and generation of multiple effector mutants is often required to affect virulence [54,57,71,72], several single knockout mutants of non-T3E HrpL-regulated genes in P. syringae DC3000 and P. savastanoi displayed reduced virulence on Arabidopsis and beans [18,73]. In general, little is known about the non-T3E genes in HrpL regulons, but homology provides reasonable scenarios for several that we identified, and we functionally validated others (below).

Among our collection of diverse HrpL-regulated, non-T3E genes, none are present in the HrpL regulon of all six strains tested, and nearly all are distributed in a mosaic pattern among the genomes of available strains (Figure 5).

PSPTO_0370 and orthologs encode a MATE efflux transporter present in an operon with iaaL which is involved in auxin conjugation to IAA-Lys [74]. Porcurated_02977 encodes a putative indole-3-glycerol phosphate synthase. Both potentially alter auxin signaling and could interfere with the balance between immune response and growth and development [75].

Several other putative transporters were identified as HrpL-regulated. PSPTO_2691 encodes a putative membrane protein TerC; PSPTO_0871 a putative macrolide efflux protein; Porcurated_01635 a putative threonine efflux protein; and PSPTO_0838 a putative major facilitator family transporter. Co-regulation of putative transporters with the T3SS suggests that promotion of nutrient acquisition, export of secondary metabolites, or detoxification of plant-encoded antimicrobials are important features of the virulence regulon.

PSPTO_0834, encoding a putative alcohol dehydrogenase, is the first gene of a putative operon comprising five genes (up to PSPTO_0838). This operon includes genes of unknown function, genes encoding a putative bifunctional deaminase-reductase enzyme and a transporter. The function of this operon remains unknown but at least PSPTO_0834 is required for full virulence of P. syringae DC3000 on Arabidopsis [18].

The PSPTO_0873-0875 putative operon is widely distributed across Pseudomonas and Erwinia species and also present in Pantoea stewartii pv. stewartii DC283. In Erwinia and P. stewartii, this operon is physically linked to the T3SS and is HrpL-regulated. PSPTO_0873 is a putative amidotransferase that makes ornithine and homocysteine from arginine and lysine. Ornithine or homo-arginine may be then incorporated into a tri- or di-peptide natural product generated by the rest of this operon. Most interestingly, hscC, hscB, hscA from Erwinia amylovora, corresponding to PSPTO_0873-0875, are required for full virulence on apple shoots [76].

PSPTO_2105 and orthologs encode a protein similar to ApeE from Salmonella typhimurium involved in thiamine biosynthesis, and was implicated in iron-sulfur cluster biosynthesis/repair, as well as FAD binding [77–79] suggesting a role during oxidative stress [70]. PSPTO_2105 is required for full virulence of P. syringae DC3000 on Arabidopsis [18]. We extend this finding by showing that the P. syringae P3HR1_1853 ortholog of PSPTO_2105 is required for full virulence of P. syringae strain 1448A on French bean (Figure S9). PSPTO_2130 and orthologs encode LuxR family DNA-binding response regulators that may be involved in regulation of regulons downstream of HrpL. Our deletion mutants of this gene in P. syringae DC3000 and P. syringae strain 1448A, or of the entire PSPTO_2130 operon, did not alter growth on Arabidopsis or French bean cv. Tendergreen, respectively (data not shown), undermining the probability of a necessary function during plant colonization in our experimental conditions. However this operon is conserved across Pseudomonas, and P. fluorescens 2973, the ortholog of PSPTO_2129 from P. fluorescens SBW25, was identified as a plant-induced gene [80]. It therefore remains plausible that this operon is involved in plant association.

Porcurated_04644 appears to encode a putative RNA N-methyltransferase, while the hypothetical protein Porcurated_03530 has homology to FliB which, in Salmonella, is responsible for methylation of flagellin [31]. We speculate that both may be involved in modification of conserved molecules known to induce host defense responses [82–84]. avd is widely distributed across bacteria and is involved in the synthesis of syringolides [85]. Syringolides are elicitors of cell death in soybean expressing the Rpe4 disease resistance gene [86,87]. The putative function of avd is discussed below.

The group II strains have smaller HrpL regulons

One of our most striking comparative observations is the relatively small size and diversity of the HrpL regulons of the phylogenetic group II strains P. syringae B728a and Pja. We observed that most of the non-T3E genes known to be HrpL-regulated in other strains are not present, or lack HrpL-regulation in group II strains, underpinning the conclusion that the limited regulon observed for P. syringae B728a and Pja can most likely be generalized to all group II strains (Figure 5). They also contain fewer T3Es than the other clades [3]. The group II strains carry genes for phytotoxins not shared by other P. syringae groups. Expression of these phytotoxins is not regulated by HrpL, and could compensate for missing T3E functions, making a smaller T3E repertoire sufficient to suppress plant defenses [3].

Modes of recruitment of non-T3E genes into, and out of, the HrpL regulon

Turnover within the HrpL regulon is known to be influenced by gene gain and loss, mediated by association of genes within the regulon with mobile elements and horizontal gene transfer (data not shown, Figure 6 A, B). However, we also observed that all the group II strains analyzed here have polymorphisms in the hrpZ-box sequence that correlated with the loss of HrpL-dependent regulation of PSPTO_2105 and orthologs (likely encoding AbpE). Several different polymorphisms within the hrpZ-box were observed, suggesting independent mutational events (Figure 4). Additionally, the group II strain orthologs of PSPTO_2130 (LuxR family), carry nucleotide polymorphisms in the consensus hrpZ-box, and are not HrpL-regulated (Figure 4). Orthologous genes from Por also display nucleotide variation in this hrpZ-box, also leading to loss of HrpL-regulation. The substitution patterns of these alterations suggest multiple, independent losses of HrpL-regulation. PSPTO_2130 and its orthologs are part of an operon where the consensus hrpZ-box is embedded within the first ORF in this operon (Figure S8) and is thus likely to be constrained by the genetic code. Interestingly, PSPTO_2130 and its orthologs have variation in the second half of the hrp box where CCAC is replaced by TCAC.
This hrc-box motif, while uncommon, is also found in PSPPH_A0106-A0112 (hopG1p), and Php_00002080 (hopC1p), each of which we define as HrpL-regulated.

The promoter erosion we observe could be driven by negative host selection pressure, or weak selection for maintenance of HrpL regulon membership combined with subsequent drift. Similarly, reversion of at least the SNPp could quickly recruit genes back into the HrpL regulon. Because the ORFs have not accumulated stop mutations, these promoter mutations are either relatively recent or there is active maintenance of the ORF sequence, perhaps for expression under different conditions.

Horizontal transfer or other types of recombination could explain how 5 regions diverge and how these regions and associated genes are recruited into the HrpL regulon. PsPam_Pam_02977, 01635, and 04371, encode an indole-3-glycerolphosphate synthase, a putative threonine efflux transporter and a hypothetical protein, respectively, that are HrpL-regulated. Similar genes are present in Psa and PjB728a, but are not HrpL-regulated (Figure 5). Putative hrc-boxes can be identified in all three Por genes, but not for the corresponding genes in Psa and PjB728a. These genes are not syntenic (data not shown). They display high similarity in their coding sequence (data not shown); however their corresponding 5' upstream regions are highly divergent. This could be the result of horizontal transfer, though there is no obvious footprint of mobile element DNA, or independent recombination events.

Lastly, loss of transcription termination regulation could lead to read-through transcription, and thus provide a mechanism for recruitment of non-T3E genes into the HrpL regulon. This mechanism was first highlighted by the recruitment into the HrpL regulon of the corR gene which was recombined downstream of the hrc-box associated hrcAQ1 gene, in PjB728a [25]. We observed that several genes found differentially expressed in our analysis were located downstream of HrpL-regulated T3E genes (Table S3) and could potentially be recruited into the HrpL regulon via loss of transcription termination regulation and subsequent transcriptional read-through.

Recruitment of a novel gene cluster into an avrD-containing virulence operon in Php1448A

We identified a cluster of HrpL-regulated genes, PSPPH_A0106-A0112, downstream from avrD that were recruited into a novel HrpL-regulated operon transcribed from the avrD promoter. These genes are flanked in Php107 and Php1448A by transposable elements, suggesting that they could be acquired by horizontal gene transfer (Figure 6). Deletion mutants of either PSPPH_A0107 or avrD resulted in reduced virulence on French bean. The slightly reduced virulence we observe is in conflict with observations that allelic replacement of avrD by the nptII gene did not result in any growth defect in complement assays [72]. This discrepancy could be explained by transcription from the nptII promoter in the previous work, or by the use of different growth assays, time points, and bean cultivars.

The PSPPH_A0106-A0112 operon is most likely involved in small molecule(s) synthesis promoting bacterial growth on host plants. Component(s) synthesized by the products of this operon and their effect on plants remain to be determined. However, since syringolides can be made from AvrD-expressing E. coli, and since the PSPPH_A0106-A0112 operon is not present in E. coli, we speculate that that the PSPPH_A0106-A0112 operon is not required for syringolide production. When present, AvrD shares no less than 54% amino acid identity across P. syringae strains. Genes encoding an AvrD-like protein with about 30% identity are widely distributed among bacteria, including Bacillus, Streptomyces and Vibrio. In general, these avrD-like genes are not found as singletons, but instead are linked to genes encoding various enzymes not related to any of the PSPPH_A0106-A0112 genes. In Streptomyces coelicolor A3(2), AvrD is part of an many mon operon responsible for synthesis of methylenomycin [30]. The PSPPH_A0110 to PSPPH_A0112 locus and to some extent the PSPPH_A0106 genes have similarity to genes in operons from Xanthomonas, Acidianus, Pectobacterium and Ralstonia. Only the Ralstonia solanacearum PS107 megaplasmid, carries both an avrD-like gene and a PSPPH_A0110-A0110 cluster of genes, but they are not contiguous on this plasmid. PSPPH_A0112 is mainly linked to P. syringae, but shares some homology with HMPREF9336_01000 (29% amino acid identity) found in Segniliparus rugous ATCC BAA-974, an opportunistic pathogen associated with mammalian lung disease [89]. HMPREF9336_01000 and an avrD-like gene are linked in Segniliparus rugous, being separated by only two genes and encoded on the same strand. We additionally observed that this operon has been disrupted by insertion of a transposable element in P. syringae CC1629, reminiscent of transposon disruptions of T3E genes commonly observed across the P. syringae phylogeny [3].

hrpL is widely distributed, and tightly linked in all hop/hrp group I T3SS [1] and the non-canonical T3SS found in some P. syringae, as well as the T3SS of P. viridiflava, P. fluorescens, Erwinia, Puntna stewartii, and Dickeya. It is the key virulence regulator in most if not all of these species. Our work highlights the advantages of integrating next generation transcriptional and genomic data to better understand the role of non-T3E HrpL regulon genes in plant-pathogen interactions. Our approach is readily applied to strains with sequenced genomes and broad phylogenetic sampling [63] to better understand P. syringae virulence mechanisms and their evolution.
Preparation of samples for RNA-seq analysis

*Pseudomonas* strains containing pBAD::hrl$_{\text{native}}$ or pBAD::EV were grown overnight at 28°C, in KB media supplemented with tetracycline, then sub-cultured in fresh media at OD$_{600}$ = 0.2, and grown until OD$_{600}$ = 0.4–0.5. Bacteria were washed twice with 10 mM MgCl$_2$ and resuspended in minimal medium [48] (MM is 50 mM KPO$_4$ pH 5.7, 7.6 mM (NH$_4$)$_2$SO$_4$, 1.7 mM MgCl$_2$, 1.7 mM NaCl) containing 10 mM mannitol and supplemented with 1% glycerol and 0.1% peptone which suppresses hrl$_{\text{L}}$ induction. Bacteria were then inoculated in supplemented minimal media at OD$_{600}$ = 0.2, and incubated shaking for 30 min at 28°C. Expression of hrl$_{\text{L}}$ was induced by addition of 200 mM L-arabinose. Aliquots of cell culture were taken 1, 3, 5 hours post-induction and treated with RNAprotect reagent (Qiagen). RNA isolation was performed by using the RNeasy mini kit (Qiagen). Isolated total RNA was treated twice with TURBO DNase (Ambion). cDNA were prepared from ribosomal depleted RNA, using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). Second strand cDNA was prepared using DNA polymerase I and Ribonuclease H (Invitrogen). Double stranded cDNA was purified using Qiaquick spin columns (Qiagen) and eluted with EB buffer. Double stranded cDNA was sheared using a Covaris Disruptor. Library was prepared according to the manufacturer’s protocol (Illumina). Sequencing of the library was performed according the manufacturer’s protocol on either Illumina GAII including single-end, 70 cycles or Illumina HiSeq 2000 including single-end, 70 cycles.

Sequence mapping and analysis

We analyzed our RNA reads using the GENE-counter pipeline. For the *P. DC3000*, *P. B728A*, and *Pph1448A* datasets, we used the publically available genomes provided by NCBI, along with the transcriptome constructed by NCBI’s gene prediction pipeline. For the *Por*, *Psy* and *Pfa* datasets, we used in-house assembly for the genome and used JGI’s Integrated Microbial Genomes – Expert Review gene prediction pipeline for the transcriptome. All ribosomal RNA genes were excluded from the transcriptome file for all datasets. Transcriptome sequences for each strain were blasted against their corresponding genome and GFF files were constructed from the Blast reports using an in-house script. We processed the RNA reads and aligned the reads using the default parameters of GENE-counter’s CASHX read mapping algorithm. Reads mapping to multiple genomic locations were excluded. Annotated genes were included in the analysis only if at least one read in one sample matched that gene which can lead to highly duplicated genes not being considered. The false discovery rate cutoff for determining differential expression was set to 0.05. We made a small modification to GENE-counter’s findDGE.pl script that allowed for random seeding during the sample depth normalization process. By repeating the normalization process 300 times we generated B-values to measure and control for normalization effects. The GenBank accession (http://www.ncbi.nlm.nih.gov/) and Gold ID (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) of the genomes used in this study are CP000058-CP000060, GI44410, CP000075, GI07003, GI03478, and AE016853-AE016855. RNA-seq data have been deposited in NCBI Gene Expression Omnibus and will be accessible through GEO Series accession number GSE46930 (http://www.ncbi.nlm.nih.gov/geo/).

Manual curation of data set and designation of Hrl$_{\text{L}}$ regulon

First, protein sequences of genes found up-regulated in our analysis with B-values$\geq$50% were used to search each genome used in this study with BlastP to identify genes split up in different contigs/scaffolds. Possible duplication was ruled out by comparing the size of the query to the size of the subject sequence (of complete genomes, principally). Putative sequencing errors leading to stop codons and discontinuous ORFs, led to consecutive queries matching the same subject sequence. Only the entry with the most significant q-value was kept. Secondly, genes encoding open reading frames shorter than 60 amino acids were excluded from our data set. Thirdly, loci of genes not previously found Hrl$_{\text{L}}$-dependent were assessed for linkage to genes with a hrl$_{\text{L}}$-box. As previously described [41,90], we observed potential transcriptional read-through artifacts for which directly Hrl$_{\text{L}}$-targeted genes led to apparent up-regulation of adjacent genes. Therefore, genes found differentially expressed adjacent to a Hrl$_{\text{L}}$-regulated gene, but on the opposite DNA strand were considered to be putative transcriptional read-through and removed from our analysis. Genes encoded on the same strand as the Hrl$_{\text{L}}$-regulated gene were kept. Fourth, genes with a hrl$_{\text{L}}$-box embedded within their ORF on either sense or anti-sense strands were not included. Adjacent genes encoded on the same strand as the manually predicted hrl$_{\text{L}}$-box were included in the defined Hrl$_{\text{L}}$ regulon, but genes on the opposite strand of the hrl$_{\text{L}}$-box were excluded. All genes removed from the Hrl$_{\text{L}}$ regulons after manual curation are listed Table S4.

Quantitative RT-PCR

For native gene expression, bacteria were grown for 4 hours in KB media from OD$_{600}$ = 0.2, washed twice with sterile 10 mM MgCl$_2$ and transferred into MM minimum media containing 10 mM mannitol for *P. DC3000* , *P. B728A*, *Pf*, *Por* strains or MM minimum media containing 10 mM fructose for *Pph1448A* strain. Cells were collected after 2 hours of incubation shaking at 28°C and treated with RNAProtect reagent (Qiagen). Total RNA derived from cells grown in MM media or arabinoinducing media (as above) was extracted using the RNeasy mini kit (Qiagen), DNase treated twice (Ambion Turbo DNase), and cleaned up with EB buffer. Double stranded cDNA was sheared using a Covaris Disruptor. Library was prepared according to the manufacturer’s protocol (Illumina). Sequencing of the library was performed according the manufacturer’s protocol on either Illumina GAII including single-end, 76 cycles or Illumina HiSeq 2000 including single-end, 70 cycles.

$\Delta$79avrRpt2-based translocation assay

Four week old Col-0 and Col-0 *avr2-101c (avr2)* plants were hand inoculated with *P. DC3000* [57] carrying $\Delta$79avrRpt2 fusion clones at OD$_{600}$ = 0.1. Plants were scored for Hypersensitive Response (HR) and pictures were taken 24 h after inoculation.

Generation of *P. syringae* knockout mutants

Knockout constructs were generated using MTN1907, a modified version of pLVC-D which allows for SacB counter-selection [3,91]. To create *Pph1448A*Δ*PSPPH A0113* mutants, 5’ and 3’ regions flanking the gene of interest were amplified using *Py* (Invitrogen) and combined by overlap extension PCR (Table S9), then cloned into pENTR-D-TOPO and sequenced. To generate the *Pph1448A*Δ*PSPPH A1855, Pp3720*Δ*hrlL* and *PorAhrpL* mutants, nucleotide sequences corresponding to the fused flanking regions of each gene were synthesized including Gateway recombination
sites and cloned in the pUC17 vector (GenScript). All five clones were recombined into MT1907 and transformed into either Pbh1448A, PPsy729 or Por by tri-parental mating. After selection on tetracycline plates, merodiploids resulting from homologous recombination were verified by PCR. Two independent merodiploids carrying either a 3’ or 5’ insertion were grown on KB agar containing 5% sucrose to select for the loss of subB via a second recombination event. Putative clean-deletion mutants were verified by PCR using flanking primers and gene specific primers.

Bacterial growth on French bean

Before inoculation, Pbhl448A and mutants were grown overnight and sub-cultured from OD600 = 0.2 for 4 hours in KB media, then washed twice with 10 mM MgCl2. Two week old French bean cv. Tendergreen improved (Livingston Seed Co.) were dip inoculated with freshly grown bacteria at OD600 = 0.001 bacteria in 10 mM MgCl2 and 0.04% Silwet L-77. Four plants were dip inoculated for each strain. Three days and an half after inoculation leaf discs were cored (12 to 16 replicates, each 4 cores), ground in 10 mM MgCl2, serially diluted and plated on KB/50 µg/ml rifampicin and bacteria counted. Each set of mutants were tested side by side with the wild type strain at least 3 times.

Supporting Information

Figure S1 Graphical representation of our experimental pipeline. Isogenic P. syringae strains carrying either pBAD::EV or pBAD::hrpL were grown on MM media supplemented with arabinose and collected 1, 3 and 5 hours post induction. RNA was extracted for each time point and cDNA prepared to confirm induction of hrpL and hrcC for P. syringae pBAD::hrpL. Total RNA for each time point was pooled equally. Pooled RNA for each strain was subjected to rRNA removal and double stranded cDNA for each time point was pooled equally. Pooled RNA for each strain was subjected to rRNA removal and double stranded cDNA prepared (Materials and Methods). Illumina libraries were prepared according to manufacturer’s protocol and sequenced. Resulting reads were used to run GENE-counter. After 300 bootstrapst of GENE-counter, genes from each sample were assigned a median read count, a median p and q-value as well as a B-value.

(TIF)

Figure S2 Detailed results of qRT-PCRs described in Table 3 from samples derived from the pBAD system. (A) For PPDc3000 genes, (B) for Pbhl1448A genes, (C) for PPsy729A genes, (D) for Por genes. cDNA was prepared from the same total RNA used to generate our RNA-seq data for all strains except PPDc3000. For PPDc3000, cDNAs were prepared from an independent biological replicate. Expression was normalized to gap-1. For determination of the relative expression, each EV sample was set to 1 and HrpL samples normalized to the corresponding EV samples. Error bars represent SD.

(TIF)

Figure S3 Detailed results of qRT-PCRs described in Table 3 from samples derived from isogenic strains grown in MM media. (A) For PPDc3000 genes, (B) for Pbhl1448A genes, (C) for PPsy729A genes, (D) for Por genes. cDNA was prepared from wild type strains and corresponding isogenic ΔhrpL mutants grown in MM media for 5 hours. Expression was normalized to gap-1. For determination of the relative % expression, each wild type strain sample was set to 100% and ΔhrpL samples normalized accordingly. Error bars represent SD.

(TIF)

Figure S4 The majority of effector genes are found up-regulated by RNA-seq. Underlined, effector genes HrpL-dependent according to Pseudomonas syringae Genome Resources, (PPI database http://www.pseudomonas-syringae.org/) but not found up-regulated in our analysis. In grey, effector genes previously identified according to a combination of homology and functional criteria described in Chang et al., 2005 and Baltrus et al., 2011 but not found to be HrpL-regulated in these strains in any experiment, to our knowledge. * indicates insertion or truncation according to PPI database. The new Por type III effectors defined in this study are listed in red.

(TIF)

Figure S5 hopBH1 and hopBI1 are both present across phylogenetically diverse strains of P. syringae. (A) Bayesian phylogenetic tree of 45 Pseudomonas strains [3,40,63] based on seven conserved loci as described in [3]. Bayesian posterior probabilities are displayed on the phylogeny only at nodes where these values are <0.95. Each phylogenetic group [defined according to Berge et al., personal communication and [27]] is color coded. (B) Distribution of hopBH1 and hopBI1 across the 45 Pseudomonas strains. Dark blue boxes indicate presence of corresponding full length ORF. Light Blue box indicates truncated ORF. White boxes indicate absence of corresponding ORF in the sequenced genome.

(TIF)

Figure S6 Amino-acid sequence alignment of HopBH1. Alignment performed using clustal W with sequences from all P. syringae strains known to date to contain hopBH1.

(TIF)

Figure S7 Expression of PSPTO_2105 and 2130 or their orthologs under native conditions supports our results obtained with arabinose-inducible hrpL system. (A) Relative expression of PSPTO_2130 and its orthologs. (B) Relative expression of PSPTO_2105 and its orthologs. qRT-PCR analysis was performed on RNA samples derived from wild type strains and the cognate isogenic ΔhrpL mutant grown in MM media. Expression was normalized to gap-1. For determination of the relative expression, each EV sample was set to 1 and HrpL samples normalized to the corresponding EV samples. Error bars represent SD.

(TIF)

Figure S8 HrpL-dependent up-regulation of genes downstream of PSPTO_2130 and its orthologs in PPDc3000. Pbhl1448A but not PPsy729A (A). Graphical representation of PSPTO_2131-PSPTO_2128 operon. qRT-PCR analysis was performed on RNA samples derived from PPDc3000(pBAD::hrpL) and PPDc3000(pBAD::EV). (C) Pbhl1448A(pBAD::hrpL) and Pbhl1448A(pBAD::EV), (D) PPsy729A(pBAD::hrpL) and PPsy729A(pBAD::EV). ORF nomenclature for operons from Pbhl1448A and PPsy729A in (C and D, respectively, is listed directly under the corresponding ORF numbers in PPDc3000 in B. Expression was normalized to gap-1. For determination of the relative expression, each EV sample was set to 1 and HrpL samples normalized to the corresponding EV samples. Error bars represent SD. qRT-PCR analysis was performed on RNA samples derived from PPDc3000 and PPDc3000(shpl). (F) Pbhl1448A and PPsy729A(shpl). For determination of the relative % expression, each wild type strain sample was set to 100% and Δshpl samples normalized accordingly. Error bars represent SD.

(TIF)

Figure S9 Pbhl1448A mutants deleted in thiamine biosynthesis lipoprotein gene display reduced growth on Tendergreen beans. Two week old bean cv. Tendergreen beans were dip inoculated with wild type Pbhl1448A (Pbh) or two independent mutants with a clean deletion of PSPL_1853 (PbhA1855 #1, PbhlA1855 #2), at
OD_{600} = 0.001. Bacterial growth of each strain was determined after 3.5 dpi. Error bars represent SD. This experiment was repeated twice with similar results.

**(TH)**

**Table S10** Determination of the relative expression of *avrD* in various *PsbA* mutants. qRT-PCR was performed on cDNA derived from wild type *PsbA* mutants, two independent clean deletions of *avrD* (#1, #2), and two independent clean deletions of *PsbA* mutants (*A0107 #1, A0107 #2*). Expression was normalized to *gap*.-1. For determination of the relative expression, each cDNA sample was set to 1 and HrpL samples normalized to the corresponding EV samples. Error bars represent SD. This experiment was repeated 3 times with similar results.

**(TH)**

**Table S1** Raw bootstrapped GENE-counter results for each strain.

**Table S2** Genes found down-regulated by RNA-seq.

**Table S3** Our defined HrpL regulons across various *P. syringae* after manual curation.

**Table S4** List of genes excluded from analysis after manual curation.

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