

## REVIEW

# Closing the Circle on the Discovery of Genes Encoding Hrp Regulon Members and Type III Secretion System Effectors in the Genomes of Three Model *Pseudomonas syringae* Strains

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*Pseudomonas syringae* strains translocate large and distinct collections of effector proteins into plant cells via the type III secretion system (T3SS). Mutations in T3SS-encoding *hrp* genes are unable to elicit the hypersensitive response or pathogenesis in nonhost and host plants, respectively. Mutations in individual effectors lack strong phenotypes, which has impeded their discovery. *P. syringae* effectors are designated Hop (Hrp outer protein) or Avr (avirulence) proteins. Some Hop proteins are considered to be extracellular T3SS helpers acting at the plant-bacterium interface. Identification of complete sets of effectors and related proteins has been enabled by the application of bioinformatic and high-throughput experimental techniques to the complete genome sequences of three model strains: *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, and *P. syringae* pv. *syringae* B728a. Several recent papers, including three in this issue of *Molecular Plant-Microbe Interactions*, address the effector inventories of these strains. These studies establish that active effector genes in *P. syringae* are expressed by the HrpL alternative sigma factor and can be predicted on the basis of *cis* Hrp promoter sequences and N-terminal amino-acid patterns. Among the three strains analyzed, *P. syringae* pv. *tomato* DC3000 has the largest effector inventory and *P. syringae* pv. *syringae* B728a has the smallest. Each strain has several effector genes that appear inactive. Only five of the 46 effector families that are represented in these three strains have an active member in all of the strains. Web-based community resources for managing and sharing growing information on these complex effector arsenals should help future efforts to understand how effectors promote *P. syringae* virulence.

**Additional keywords:** ECF sigma factor, functional genomics, microarrays.

*Pseudomonas syringae* represents a group of economically important bacterial pathogens noted for their diverse interactions

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with plants (Hirano and Upper 2000). Pathovars of *P. syringae* vary in their capacity for epiphytic survival, the nature of the symptoms they elicit, and their host range. The molecular basis for these interactions appears complex because it is controlled by the interplay of several classes of factors and each class can have many members with overlapping functions. Classes of virulence factors include phytotoxins, phytohormones, adhesins, cell-wall degrading enzymes, and most importantly, effector proteins translocated into plants by the type III secretion system (T3SS) (Buell et al. 2003). The T3SS is also known as the Hrp system because it is encoded by *hrp* genes that are required for *P. syringae* strains to elicit the hypersensitive response (HR) in nonhosts or to be pathogenic in hosts. Also modulating bacterium-plant interactions are classes of factors contributing to plant defense, including microbe-associated molecular patterns, which are produced by the bacteria and corresponding pattern-recognition proteins that are produced by the plant, as well as resistance proteins that recognize effectors or their activity within plant cells (Alfano and Collmer 2004; Chisholm et al. 2006; Grant et al., in press; Mudgett 2005; Nomura et al. 2005). Identifying the complete set of factors in each class is prerequisite to deconvoluting the molecular basis and evolution of *P. syringae*-plant pathosystems.

The study of *P. syringae* virulence has been revolutionized by genomics, beginning with the publicly available draft sequence and then the complete sequence of *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003), followed by the complete sequences of *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *syringae* 1448A (Feil et al. 2005; Joardar et al. 2005). These three strains represent an attractive set for functional genomics. They have different host ranges and pathogenic strategies and they represent each of the three major phylogenetic clades that contain the 50 or so pathovars within this species (Sarkar and Guttman 2004; Sawada et al. 1999). Each strain provides a different window into the complex interactions of *P. syringae* with plants. *P. syringae* pv. *tomato* DC3000 causes bacterial speck of *Arabidopsis* and tomato and has emerged as a model for studying basic virulence mechanisms (Preston 2000; Quirino and Bent 2003). *P. syringae* pv. *phaseolicola* causes halo blight of bean, an important disease in several developing countries, and is a model for studying the nature and variability of race-cultivar interactions (Jackson et al. 1999; Pitman et al. 2005). *P. syringae* pv. *syringae*, which causes brown spot of bean, is a

model for studying bacterial epiphytic growth and behavior in the field (Hirano and Upper 2000; Marco et al. 2005; Monier and Lindow 2003).

Type III effectors provided the ideal demonstration case for applying functional genomics to the study of *P. syringae* virulence because of the collective importance and large number of these proteins. Consequently, several labs around the world have participated in genome-enabled identification of type III effectors in *P. syringae*. Two previous reviews describe the first phase in this effort, which was based on draft-phase *P. syringae* pv. *tomato* DC3000 genome sequences (Collmer et al. 2002; Greenberg and Vinatzer 2003). This short review focuses on several recent papers, including three in this issue, that exploit complete genome sequences to explore the Hrp regulons and effector inventories of *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, and *P. syringae* pv. *syringae* B728a. These papers report a differential fluorescence induction (DFI) screen for effectors in DC3000 and 1448A (Chang et al. 2005), identification and validation of multiple effectors in B728a (Vinatzer et al. 2005), microarray analyses of the Hrp regulon in DC3000 (Ferreira et al. 2006; Lan et al. 2006), multiple approaches to a complete inventory of the effector genes of DC3000 (Schechter et al. 2006; Vinatzer et al. 2005), and the facile use of bioinformatic methods to comprehensively identify effector genes in DC3000 and 1448A (Ferreira et al. 2006; Vencato et al. 2006). Importantly, these papers illustrate the power of functional genomics in the study of complex virulence systems and highlight the value of curated, web-accessible, community datasets as a foundation for future work.

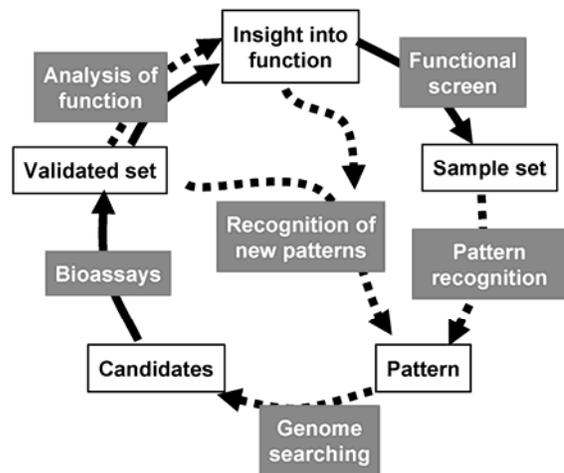
**The bioinformatic-enabled candidate gene approach permits identification of complete sets of virulence genes without a prior requirement for evident phenotypes.**

The development of transposon mutagenesis tools in the mid-1980s provided a method for tagging and cloning virulence genes that was broadly applicable but limited by the requirement for a robust phenotype. Transposon-tagging easily identified the *hrp* genes encoding the T3SS in *P. syringae* because of their strong phenotypes in inoculated plants, but it failed to identify effector genes because they lacked such a phenotype. However, a sample set of effector genes had been identified in the pregenomics era on the basis of gain-of-function assays for avirulence in inoculated plants carrying cognate resistance (*R*) genes or for the ability of the encoded proteins to travel the T3SS pathway. Effectors identified by these assays were given Avr (avirulence) or Hop (Hrp outer protein) designations, respectively (Lindeberg et al. 2005). The bioinformatic-enabled candidate gene approach allowed patterns associated with this sample set to be applied to the complete genome of *P. syringae* pv. *tomato* DC3000 in search of all potential *avr/hop* effector genes (Fig. 1). This approach involves iterative cycles of pattern recognition and pattern-based genome searching followed by candidate validation. Genes in the initial sample set were characteristically preceded by “Hrp box” promoter sequences that are responsive to the HrpL alternative sigma factor (Fouts et al. 2002; Guttman et al. 2002; Zwiesler-Vollick et al. 2002). As the set of validated effector proteins grew, patterns associated with their N-terminal 50 amino acids were recognized and were then used as an additional search tool (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002).

The use of promoter sequences and targeting-associated patterns to search the draft genome of DC3000 demonstrated that these patterns could identify many effector candidates, and subsequent testing validated several of these (Collmer et al. 2002; Greenberg and Vinatzer 2003; Schechter et al.

2004; Vinatzer et al. 2005). However, many questions remained. Can bioinformatic methods, such as hidden Markov modeling, predict all HrpL-response promoters? Are all active effector genes in *P. syringae* preceded by Hrp promoters? Do all effector proteins possess targeting-associated patterns (or linkage with chaperones that are characteristic of some effectors or both)? Can these bioinformatic features be used to comprehensively and selectively identify all of the active effectors in any *P. syringae* strain for which there is a genome sequence?

There are several reasons why these questions persisted. First, there are multiple variations in validated Hrp promoter sequences but the limits of permissible variation have not been determined. Second, the process by which T3SS substrates are targeted to the pathway is still poorly understood and an actual function for the *P. syringae* targeting-associated patterns awaits demonstration. Third, in many other T3SS-dependent plant and animal pathogens, some effector genes are regulated independently of the T3SS genes and some effectors have N-termini that are completely devoid of the patterns associated with *P. syringae* T3SS substrates. This raises the possibility that these patterns can be used to find only a subset of the *P. syringae* effectors. Furthermore, as the inventory of candidate effectors grows, new concerns arise regarding the possibility of false positives or of effectors that are real but are not actively deployed by the studied strain. Resolving these issues required genome-enabled, high-throughput experimental approaches that identify Hrp regulon members and effectors independently of promoter and targeting-associated patterns. As will be discussed next, several of the papers highlighted in this short review achieve that end and thereby permit rigorous assessment of the bioinformatic approach.



**Fig. 1.** The iterative, bioinformatic/experimental, pattern-based approach to finding and validating candidate genes in multifactorial systems in bacterial genomes. The representative cycle of experimental steps (solid arrows) and bioinformatic steps (dashed arrows) was used to identify type III effector genes in *Pseudomonas syringae* pv. *tomato* DC3000 and then *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* B728a. In the first step, gain-of-function screens for avirulence phenotypes yielded a sample set of *P. syringae* effectors. Hrp box promoter patterns were discerned upstream of these genes. Hidden Markov modeling of an expanded sample set of *P. syringae* pv. *tomato* DC3000 Hrp promoters enabled comprehensive searching of the genome for candidate effector genes. Translocation bioassays validated effectors. New patterns associated with the N-terminal amino acids of validated effectors were discerned and were used in combination with Hrp promoters to efficiently identify high-probability effector candidates. Further analysis of large sets of effectors can yield insights into effector evolution, active domains, and new patterns underlying functions in pathogenesis.

**Comprehensive experimental identification of HrpL-responsive genes in *P. syringae* pv. *tomato* DC3000 validates the bioinformatic approach to Hrp promoter identification.**

The transfer of *P. syringae* to a minimal medium that mimics plant intercellular fluids triggers a regulatory cascade involving the sequential action of genes encoding the HrpRS  $\sigma$ 54 enhancer binding proteins, the HrpL extracytoplasmic factor (ECF) sigma factor, and finally, the T3SS machinery and effectors. A review in this Focus Section provides more background on factors controlling HrpL activation (Tang et al. 2006). The emphasis here is on effectors and other genes that are activated by HrpL. Two approaches have been employed to comprehensively identify such genes using high-throughput experimental methods. Chang et al. (2005) used DFI and a fluorescence-activated cell sorter (FACS) to iden-

tify operon fusions that are upregulated following inducible overexpression of HrpL. Sequencing of many clones following FACS enrichment for HrpL activation resulted in a near-saturation screen. Alternatively, Lan et al. (2006) and Ferreira et al. (2006) used microarrays to identify genes that are differentially expressed in minimal medium in wild-type DC3000 when compared with *hrpL* mutant strains. The three reports yield congruent results for strongly expressed T3SS machinery and effector genes, but differences in the studies yield additional insights into the HrpL regulon. For example, the DFI screen was also performed on *P. syringae* pv. *phaseolicola*, which enabled HrpL regulon comparisons that are discussed below. Lan et al. (2006) included a microarray analysis of genes that are activated by HrpRS, and Ferreira et al. (2006) featured a kinetic analysis of HrpL-dependent gene activation.

**Table 1.** Genes encoding effectors and related proteins in *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000, *P. syringae* pv. *phaseolicola* (*Pph*) 1448A, and *P. syringae* pv. *syringae* (*Psy*) B728a<sup>a</sup>

Family/subfamily <sup>a</sup>	Previous names <sup>b</sup>	<i>Pto</i> DC3000 <sup>c</sup>	<i>Pph</i> 1448A	<i>Psy</i> B728a	Family/subfamily <sup>a</sup>	Previous names <sup>b</sup>	<i>Pto</i> DC3000 <sup>c</sup>	<i>Pph</i> 1448A	<i>Psy</i> B728a
<i>avrB2</i>	<i>avrC</i>		<b>PSPPH_A0120</b>		<i>hopY1</i>		PSPTO_0061		
<i>avrB3</i>				Psyr_1219	<i>hopZ3</i>				Psyr_1224
<i>avrB4-1</i>			PSPPH_3028		<i>hopAA1</i>				Psyr_1183
<i>avrB4-2</i>			PSPPH_0784		<i>hopAA1'</i>			PSPPH_1263	
<i>avrD1</i>	<i>avrD</i>		<b>PSPPH_A0113</b>		<i>hopAA1-1</i>		PSPTO1372		
<i>avrE1</i>	<i>avrE</i>	PSPTO_1377	PSPPH_1268	Psyr_1188	<i>hopAA1-2</i>		PSPTO_4718		
<i>avrPto1</i>	<i>avrPto</i>	PSPTO_4001		Psyr_4919	<i>hopAB1</i>	<i>virPphA</i>		<b>PSPPH_A0127</b>	Psyr_4659
<i>avrRpm1</i>				Psyr_0738	<i>hopAB2</i>	<i>avrPtoB</i>	PSPTO_3087		
<i>avrRps4</i>			<b>PSPPH_A0087</b>		<i>hopAB3'</i>			PSPPH_2294	
<i>hopA1</i>	<i>hrmA, hopPsyA</i>	PSPTO_5354			<i>hopAD1</i>		PSPTO_4691		
<i>hopB1</i>		PSPTO_1406			<i>hopAE1</i>			PSPPH_4326	Psyr_4269
<i>hopC1</i>	<i>avrPpiC2</i>	PSPTO_0589			<i>hopAF1</i>		PSPTO_1568	PSPPH_1443	Psyr_3813
<i>hopD1</i>	<i>avrPphD</i>	PSPTO_0876	<b>PSPPH_A0010</b>		<i>hopAG1</i>				Psyr_0778
<i>hopD::IS52</i>		PSPTO_4724			<i>hopAG::ISPssy</i>		PSPTO_0901		
<i>hopE1</i>		PSPTO_4331			<i>hopAH1</i>		PSPTO_0905		Psyr_0779
<i>hopF2</i>	<i>avrPphF</i>	PSPTO_0502			<i>hopAH2</i>			PSPPH_3036	Psyr_3123
<i>hopF3</i>			PSPPH_3498		<i>hopAH2-1</i>		PSPTO_3292		
<i>hopG1</i>		PSPTO_4727	PSPPH_0767		<i>hopAH2-2</i>		PSPTO_3293		
<i>hopH1</i>		PSPTO_0588		Psyr_1889	<i>hopA11</i>		PSPTO_0906		
<i>hopH::ISPsy4</i>		PSPTO_5623			<i>hopA11'</i>				Psyr_0780
<i>hopI1</i>		PSPTO_4776	PSPPH_4366	Psyr_4326	<i>hopAM1-1</i>	<i>avrPpiB1</i>	PSPTO_1022		
<i>hopK1</i>		PSPTO_0044			<i>hopAM1-2</i>	<i>avrPpiB2</i>	<b>PSPTO_A0005</b>		
<i>hopM1</i>		PSPTO_1375		Psyr_1186	<i>hopANI</i>	<i>lpx53</i>	PSPTO_5061	PSPPH_0456	Psyr_0465
<i>hopM1'</i>			PSPPH_1266		<i>hopAO1</i>	<i>avrPphD2</i>	PSPTO_4722		
<i>hopN1</i>		PSPTO_1370			<i>hopAQ1</i>		PSPTO_4703		
<i>hopO1-1</i>		<b>PSPTO_A0018</b>			<i>hopAS1</i>			PSPPH_4736	
<i>hopO1-2</i>		PSPTO_4594			<i>hopAS1'</i>		PSPTO_0474		
<i>hopO1-3'</i>		PSPTO_4592			<i>hopAT1</i>			PSPPH_5225	
<i>hopQ1</i>			<b>PSPPH_A0012</b>		<i>hopAT1'</i>		PSPTO_5618		
<i>hopQ1-1</i>		PSPTO_0877			<i>hopAU1</i>			<b>PSPPH_A0031</b>	
<i>hopQ1-2</i>		PSPTO_4732			<i>hopAV1</i>			<b>PSPPH_A0056</b>	
<i>hopR1</i>		PSPTO_0883	PSPPH_0171		<i>hopAW1</i>			<b>PSPPH_A0122</b>	
<i>hopS1::ISPssy</i>		PSPTO_4597			Harpins, pilus-associated proteins, and other candidate T3SS helpers				
<i>hopS2</i>		PSPTO_4588			<i>hrpA1</i>		PSPTO_1381		
<i>hopT1-1</i>		<b>PSPTO_A0019</b>			<i>hrpA2</i>			PSPPH_1272	Psyr_1192
<i>hopT1-2</i>		PSPTO_4593			<i>hrpK1</i>		PSPTO_1405	PSPPH_1295	Psyr_1218
<i>hopT2</i>		PSPTO_4590			<i>hrpW1</i>		PSPTO_1373	PSPPH_1264	Psyr_1184
<i>hopU1</i>		PSPTO_0501			<i>hrpZ1</i>		PSPTO_1382	PSPPH_1273	Psyr_1193
<i>hopV1</i>		PSPTO_4720	PSPPH_2351		<i>hopP1</i>		PSPTO_2678		
<i>hopW1-1</i>			<b>PSPPH_A0009</b>		<i>hopAJ1</i>		PSPTO_0852	PSPPH_0763	
<i>hopW1-2</i>			<b>PSPPH_A0075</b>		<i>hopAK1</i>		PSPTO_4101	PSPPH_1424	Psyr_3839
<i>hopX1</i>	<i>avrPphE</i>	<b>PSPTO_A0012</b>	PSPPH_1296	Psyr_1220					

<sup>a</sup> Hop gene families are listed according to the names assigned under the new Hop nomenclature, with family and subfamily assignments based on the phylogenetic distances calculated in MEGA2.1 from CLUSTAL sequence alignments (Lindeberg et al. 2005). Heavy lines are used to group subfamilies within a single family. The nature and source of data supporting the *hop* designations can be found in the Hop Database at the Pseudomonas-Plant Interaction website. Information on the *hopJ*, *hopL*, *hopAC*, and *hopAP* families is also found there, though they are omitted from this list since no validated members are HrpL-regulated and T3SS substrates.

<sup>b</sup> In cases where *hop* names replace previously assigned names having functional significance, the earlier common names are indicated. A comprehensive list of previously assigned names for all *hops* listed can be found in the Hop Database at the Pseudomonas-Plant Interaction website. Note that *avrPto* and *avrPtoB* continue to be widely used because of extensive precedence in the literature.

<sup>c</sup> Presence of *hop* genes in the three *P. syringae* pathovars is indicated by the locus tag for the gene in question. Locus tags in bold indicate plasmid-borne genes. Locus tags are colored as follows: pink, experimental evidence supports HrpL-dependent regulation and passage through the T3SS; blue, bioinformatic and/or experimental evidence argues against HrpL-dependent expression and/or passage through the T3SS or gene is disrupted in 5' region; yellow, experimental evidence supports HrpL-dependent regulation or passage through the T3SS, but evidence is either incomplete or contradictory; white, HrpL-dependent regulation and passage through the T3SS predicted but not experimentally evaluated.

Only half of the 129 genes that are activated by HrpRS are activated by HrpL, and five of the genes that are activated exclusively by HrpRS are transcriptional regulators (Lan et al. 2006). Furthermore, several of the genes or operons that are activated by HrpL lack Hrp promoters (Ferreira et al. 2006; Lan et al. 2006). Thus, HrpRS and HrpL appear to control multiple, candidate, virulence-related subsidiary regulons. In contrast to genes that are directly regulated by HrpL, those indirectly regulated appear to be more affected by culture conditions or HrpL expression levels. For example, overexpression of HrpL in repressive media enhanced the expression of *cfl* genes directing biosynthesis of the phytotoxin coronatine (Chang et al. 2005; Fouts et al. 2002), but mutants lacking HrpL were unaffected in their expression of these genes in minimal media (Ferreira et al. 2006). The conditions and circuits controlling the ancillary regulons associated with HrpRS and HrpL are a high priority for future study, but the focus here is on the genes preceded by HrpL-responsive Hrp promoters, which we refer to as the HrpL regulon.

Hierarchical clustering based on expression kinetics revealed that the most rapidly activated genes in the HrpL regulon control extracellular components of the T3SS, such as the HrpA pilus protein, the HrpK putative translocator, and harpins, which may have a role as extracellular T3SS helpers (Ferreira et al. 2006). The rapid activation of similar genes in *P. syringae* pv. *phaseolicola* was previously observed using real-time polymerase chain reaction (PCR) (Thwaites et al. 2004). Although effectors form the largest class of genes preceded by Hrp promoters, it is important to note that nearly twenty Hrp promoter-driven genes have no apparent T3SS-related function (Ferreira et al. 2006; Lan et al. 2006). PSPTO2105 (ApeB-family protein) and PSPTO0834 (alcohol dehydrogenase) represent two of these genes, and mutations in each reduce the growth of *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* leaves (Vencato et al. 2006). Learning how these proteins contribute to bacterial growth in planta and whether the products of other non-T3SS genes in the HrpL regulon have a similar role is another important target for future research.

Many of the *P. syringae* pv. *tomato* DC3000 Hrp promoters that were experimentally validated by DFI and microarray analyses had been predicted by hidden Markov modeling, based on a preliminary training set of validated Hrp promoters (Fouts et al. 2002). The much larger set of validated Hrp promoters generated by microarray analysis enabled Ferreira et al. (2006) to rigorously assess the predictive value of both hidden Markov modeling and weight matrix modeling and to use Gibbs sampling to look for additional regulatory sequences upstream of HrpL-activated genes. Interestingly, Gibbs sampling identified no motifs other than the Hrp box. This raises the possibility that HrpL is the sole regulator of these genes. Reliance on a single regulator and *cis* regulatory element presumably would enable rapid recruitment into the HrpL regulon of horizontally acquired effector genes.

The hidden Markov and weight matrix models generate independent lists of candidate promoters ranked by descending scores, which can be further analyzed for likelihood of activity based on genomic context. All of the experimentally validated Hrp promoters have a hidden Markov model score greater than 10.0 or a weight matrix score greater than 4.0 and a distance between the promoter and apparent start site of less than 220 base pairs. The hidden Markov model is simpler to run, and a cutoff score of 10.0 captures virtually all of the validated Hrp promoters. Importantly, the hidden Markov model identifies several active Hrp promoters that were missed by one or the other of the experimental approaches, and marginal promoters found by bioinformatics are retained on the ranked list for future testing rather than being missed entirely. The overriding

advantage of the bioinformatic approach to identification of Hrp promoters in sequenced genomes is that it can be completed in a few days, and the costs are essentially those of genome sequencing, which are dropping dramatically with the development of new technologies (Margulies et al. 2005). A fundamental limitation of the bioinformatic approach is that it misses genes that are indirectly activated by HrpL. Ferreira et al. (2006) provide a bioinformatic flowplan for the identification of Hrp promoters in any sequenced strain of *P. syringae*. Below, we will show the utility of these protocols in the identification of Hrp promoters in *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* B728a.

### **Extensive testing of potential *P. syringae* pv. *tomato* DC3000 T3SS substrates validates the bioinformatic approach to identifying effector genes.**

Analyses of the *P. syringae* pv. *tomato* DC3000 genome during the draft phase revealed that many effector genes could be predicted by their linkage with Hrp promoters and possession of characteristic patterns in the N-terminal 50 amino acids of the encoded proteins (Fouts et al. 2002; Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). Three of these patterns have been widely used to predict *P. syringae* effectors: i) a serine content of at least 10%, ii) certain aliphatic amino acids in position three or four, and iii) a lack of acidic amino acids in the first 12 residues. Chang et al. (2005) and Schechter et al. (2006) take two different approaches to experimentally validating a comprehensive inventory of effectors in the completed genome of DC3000. The former report tests whether high-probability candidate effectors expressed from their native promoters on a multicopy plasmid can translocate functional levels of C-terminally-fused  $\Delta 79\text{AvrRpt2}$ . Importantly, this approach seeks evidence that native promoters are strong enough to support delivery of their respective effectors. The latter report tests the ability of 44 candidates that have varying probabilities of being effectors to translocate the *Cya* (*Bordetella pertussis* adenylate cyclase) reporter into plant cells when expressed from a *tac* vector promoter. An important goal of this report is to step outside the circle of expectations regarding *P. syringae* effectors and seek novel effectors based on potential eukaryote biochemical activities or linkage with known effector genes despite the lack of Hrp promoters and known targeting patterns. Also, important is the independent report by Vinatzer et al. (2005) validating translocation of seven of the high-probability effectors in DC3000.

In general, these studies corroborated previous bioinformatic-based predictions and observations (Fouts et al. 2002; Guttman et al. 2002; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004; Zwiesler-Vollick et al. 2002). However, the negative results are particularly interesting. First, no evidence was found for a novel class of effectors expressed independently of the T3SS or lacking the targeting-associated patterns (Schechter et al. 2006; Vinatzer et al. 2005). Second, a subset of the DC3000 effector genes are functionally inactive because of mobile genetic element insertions or other mutations that disrupt either their expression (tested by real-time PCR), their integrity, or the translocation of their products (tested as *tac*-driven *Cya* fusions) (Schechter et al. 2006). Third, some of the effector/Hop proteins that have validated Hrp promoters and deliver *tac*-driven *Cya* fusions fail to translocate functional levels of  $\Delta 79\text{AvrRpt2}$  when expressed from their native promoter (Chang et al. 2005). In summary, these observations indicate that the bioinformatic approach can identify virtually all of the effector genes in a *P. syringae* genome, but not all of these genes are functional.

With more than 50 genes encoding Hop/effectors in *P. syringae* pv. *tomato* DC3000 (Table 1), functional classification,

nomenclature, and bases for research prioritization become important issues. Here, it is useful to revisit three terms that have been used for *P. syringae* T3SS-substrates: effector, Hop, and helper. The term “effector” is prevalent because it applies to all pathogens and to the largest class of proteins traveling the T3SS for most pathogens. True effectors are considered to have their primary action within host cells (Cornelis and Van Gijsegem 2000), whereas helper (accessory) proteins appear to act at the interface between host and parasite in support of the T3SS pathway (Collmer et al. 2002). The HrpA pilin clearly has such a role, but HrpK and harpins are also thought to be helpers. Terms such as Yop (*Yersinia* outer protein) and Hop have the advantage of applying to both helpers and true effectors, and of course, they provide three-letter designations for naming bacterial genes (Lindeberg et al. 2005). Nevertheless, because the vast majority of Hops appear to be true effectors and the term effector is so widely recognized, we will use this term primarily as we turn to the issue of candidates that fail expression or translocation tests and to the possibility of false positives in the DC3000 effector inventory.

There are several reasons why candidates can fail translocation tests. First, helper proteins expressed from their native promoter appear not to efficiently translocate the  $\Delta 79\text{AvrRpt2}$  reporter (Chang et al. 2005). However, this test may not reliably differentiate helpers and true effectors. For example, HopP1- $\Delta 79\text{AvrRpt2}$  passes this translocation test, but HopP1 has many properties of harpins: the N-terminal half of the protein is highly similar to the HrpW1 harpin, the C-terminal half has a lysozyme-like lytic transglycosylase domain, and the isolated protein can elicit the HR when infiltrated into the apoplast of tobacco leaves (A. R. Ramos and A. Collmer, *unpublished data*). Resolving these uncertainties will ultimately require a better understanding of the function and site of action of harpins and other putative helper proteins. Second, some effector genes in DC3000 do not appear to be expressed or their proteins do not pass any T3SS substrate tests. However, these proteins are all members of validated Hop/effector families (Table 1). Given that loss of gene function can be important in virulence evolution and that effector loss could potentially expand or shift host range, these inactive effectors (appropriately noted as such) are an important part of the inventory. Third, although the native-promoter  $\Delta 79\text{AvrRpt2}$ -reporter test provides useful clues as to which effectors are most abundantly translocated, it cannot be used alone to determine what is or is not an effector or whether an effector is delivered at levels needed for its own function. In essence, the assay determines whether enough  $\Delta 79\text{AvrRpt2}$  is delivered to elicit an *RPS2*-dependent HR (Chang et al. 2005; Mudgett et al. 2000). However, as discussed further by Schechter et al. (2006), some effectors may interfere with HR elicitation, the assay does not determine whether a test effector is delivered at a level needed for its own activity (as opposed to the activity of AvrRpt2), and some effectors that are known to be active in DC3000 do not pass this test (Chang et al. 2005).

Table 1 contains a complete list of the 53 T3SS effector and helper genes in *P. syringae* pv. *tomato* DC3000, including those that appear inactive. The Hop names in Table 1 reflect unified nomenclature recommendations (Lindeberg et al. 2005), and further documentation for all of the data in the table is provided by Schechter et al. (2006) and in the Hop database maintained at the Pseudomonas-Plant Interaction website. Effector genes associated with *avr* designations are members of established effector families that were typically founded using strains other than DC3000. It is important to note that every effector/Hop family has at least one member in some *P. syringae* strain that is HrpL-regulated and shown to travel the

T3SS pathway, as stipulated by the unified Hop nomenclature guidelines (Lindeberg et al. 2005). This requirement is important because it eliminates false positives that may pass T3SS substrate tests. False positives potentially could be found in the flagellar proteins, which travel the flagellar biogenesis T3SS and have the same targeting-associated N-terminal amino-acid patterns as *P. syringae* effectors. Interestingly, flagellar genes are downregulated when the Hrp T3SS is activated (Ferreira et al. 2006; Lan et al. 2006). False positives in T3SS translocation tests can also be obtained with Cya reporter fusions involving N-terminal fragments rather than complete test proteins (L. M. Schechter, *unpublished results*). In summary, we think that the effector inventory for DC3000 is substantially complete and does not contain any false positives. However, as discussed further below, mutant phenotypes are ultimately needed to validate the arsenal of effectors that are active in interactions with a given plant.

#### **The techniques honed with *P. syringae* pv. *tomato* DC3000 have been extended to the HrpL regulons and effector inventories of *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* B728a.**

*P. syringae* pv. *phaseolicola* 1448A provided an ideal case for the test by Vencato et al. (2006) of the ability of the bioinformatic methods developed with DC3000 to work in another sequenced strain; 1448A is in a different pathovar and phylogenetic clade and its effector inventory was independently assessed by near-saturation DFI screening and  $\Delta 79\text{AvrRpt2}$  reporter translocation assays (Chang et al. 2005). The bioinformatic and DFI screens yielded essentially congruent results for strongly expressed Hrp promoters, but differences occurred with weaker or atypical Hrp promoters. For example, two atypical promoters detected by the DFI screen were below the hidden Markov model high-probability cutoff (although they were above the cutoff for the weight matrix model). In contrast, the DFI screen failed to detect several high-probability Hrp promoters (subsequently validated by real-time PCR), including those for two novel effectors and the 1448A ApbE protein whose ortholog in DC3000 was shown to contribute to bacterial growth in planta (Vencato et al. 2006). Subsequent identification of effector genes within the 1448A HrpL regulon based on targeting-associated patterns also yielded similar results. However, it is important to note that *hopAVI*<sub>Pph1448A</sub> and *hopAWI*<sub>Pph1448A</sub> were two effector genes found by Chang et al. (2006) that were missed during an initial draft-phase analysis of the 1448A genome because GLIMMER failed to identify the open reading frames (ORF). In general, correct identification of start codons, particularly in genomic islands, is a limiting factor in analyzing potential N-terminal targeting-associated patterns (Vencato et al. 2006). The effector inventory for *P. syringae* pv. *phaseolicola* 1448A is presented in Table 1.

The annotation of the *P. syringae* pv. *syringae* B728a genome emphasized factors underlying the epiphytic fitness of this strain rather than the HrpL regulon and effector genes (Feil et al. 2005). However, Greenberg and Vinatzer (2003) have contributed important analyses of the effector inventory of B728a (as well as DC3000), including recent demonstration of the T3SS-dependent translocation of four novel B728a effectors (Vinatzer et al. 2005). To enable comparisons of the HrpL regulons of all three of the sequenced *P. syringae* strains, we used the standardized bioinformatic protocol described by Ferreira et al. (2006) and Vencato et al. (2006) to produce equivalent data for *P. syringae* pv. *syringae* B728a. We also analyzed downstream ORFs for any candidate effectors based on N-terminal targeting-associated patterns additional to those

previously noted (Greenberg and Vinatzer 2003; Vinatzer et al. 2005). Table 1 presents the current inventory of effector genes in *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, and *P. syringae* pv. *syringae* B728a.

**The effector inventories of *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, and *P. syringae* pv. *syringae* B728a reveal that the active effectors are substantially different in each strain and many effector genes appear inactive.**

In analyzing Table 1, it is important to remember that many genes are placed in the effector inventory of a given strain through homology-based membership in an Avr or Hop family that was founded in another strain and may be active only in other strains (Lindeberg et al. 2005). Interestingly, all three of the sequenced strains contain several effector genes that appear to be inactive because of lack of a Hrp promoter, mobile element insertion, or frameshift mutation. These genes are color-coded blue in Table 1. Visualizing the pattern of blue and blank boxes against the background of boxes with likely-active effectors in Table 1 affords several observations. For example, *P. syringae* pv. *syringae* B728a has substantially fewer effectors (active or inactive) than the other two strains. Does this reflect an adaptation of B728a for epiphytic rather than endophytic growth? Or do the necrosis-inducing, pore-forming toxins of B728a substitute for some effector functions? In contrast, *P. syringae* pv. *tomato* DC3000 has substantially more effectors than the other two strains. This may underlie the ability of DC3000 to attack hosts in two different plant families. Regarding host range, it is interesting that there is relatively little overlap in the effector arsenals of *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* B728a although they are both pathogens of bean; HopAE1 is the only effector family that is present in both of these strains but lacking in DC3000. It is also striking that the T3SS helper families are more universally distributed in *P. syringae* strains than the effector families. In fact, it appears that only the AvrE1, HopI1, HopX1, Hop AB, and HopAF1 effector families are actively deployed in all three strains (the HopMI family is questionable because *hopMI*<sub>Pph1448A</sub> has a C-terminal truncation). But it should also be noted that universally distributed helpers and effectors may have important differences among strains. For example, the HrpA pilin proteins of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* belong to different subfamilies and are only 28% identical (Inoue and Takikawa 2006; Preston et al. 1995), and *hopX1* (*avrPphE*) alleles in *P. syringae* pv. *phaseolicola* races differ in their avirulence phenotypes (Stevens et al. 1998). Phylogenetic studies suggest that some effector genes, such as *avrE* and *hopX1*, are ancient within *P. syringae*, whereas others, such as *hopAMI* and *hopQ1*, have been acquired by more recent horizontal gene transfers into some strains (Deng et al. 2003; Rohmer et al. 2004).

Table 1 also highlights ongoing uncertainties regarding the status of many *P. syringae* effectors, with the yellow boxes indicating conflicting experimental data and the white boxes indicating the lack of tests for active deployment by that strain. Importantly, these assessments are based largely on effector deployment rather than effector function in pathogenesis. Because effectors such as HopAB2 (AvrPtoB) appear to have multiple functional domains (Abramovitch et al. 2003), it is possible that some effectors may be mistakenly predicted to be inactive because of truncations. Furthermore, some effector genes, such as *hopWI-1* and *hopWI-2* are not obviously disrupted but have much larger homologs in other strains, leaving it unclear whether one form represents an inactive fragment or the other an inactive fusion.

The evidence-coding in Table 1 should help prioritize future efforts to delineate the active arsenal within the total effector inventory of these three model strains. However, understanding the operation of this arsenal in pathogenesis will require additional approaches. For example, we need mutations with loss-of-function phenotypes. In this regard, it appears that polymorphants lacking combinations of effector genes will be particularly useful. Although mutation of individual effector genes may affect cell-death phenotypes in inoculated plants, mutation of two or more effector genes can substantially reduce growth in planta (Alfano et al. 2000; Badel et al. 2003, 2006; Lin and Martin 2005). Also, we need to better understand effector actions within plant cells with regard to molecular targets, biochemical activities, and parasitic benefit through defense suppression and nutrient release. Two recent reviews summarize exciting progress in this area and highlight effector redundancy at the functional level by noting that multiple effectors contribute to suppressing defense-associated responses, such as papilla formation or cell death in nonhosts (Chisholm et al. 2006; Grant et al., in press).

What forces select for the complex effector arsenals of *P. syringae* strains? An overriding consideration here is that effectors (or more likely their activity) can be detected by the *R*-gene surveillance system, and consequently, many effectors are double agents capable of suppressing defenses in one plant and eliciting them in another (Alfano and Collmer 2004; Espinosa and Alfano 2004; Nomura et al. 2005). Such effector-*R* gene interactions have long been known to limit *P. syringae* host range at the race-host cultivar level (Keen 1990), but what controls host range at the pathovar-host species level is less clear. A key question is whether the virulence targets of effectors differ among plants. If so, seemingly redundant interdiction of pathways leading to basal defenses could promote a larger host-range and enable basic virulence to be retained even if newly evolved *R*-gene recognition by one of the hosts has selected for loss of one of the effectors. Such an effector loss in the face of *R*-gene selection pressure has recently been documented (Pitman et al. 2005). An alternative (and not mutually exclusive) model is that complexity in the arsenal is driven by the need for interactions among effectors as they coordinately disarm multiple layers of plant defenses. The observation that the loss of *hopAB1* (*virPphA*) from *P. syringae* pv. *phaseolicola* 1449B unmasks cryptic avirulence activity of other effectors supports this model. Our growing knowledge of the effector arsenals of three model strains, as summarized in Table 1, provides a foundation for exploring such questions, and it highlights the need for systematic approaches as we explore the functions of this complex system.

**Community resources for *P. syringae* effector research can promote the conversion of data to new knowledge.**

The *P. syringae* T3SS effector system is highly complex. We have emphasized above the importance of pattern recognition in dealing with that complexity in the effector discovery process. Here, we will describe resources provided through the Pseudomonas-Plant Interaction website that are intended to help the community manage effector-related data and visualize new biological patterns. In that regard, our experience with effector discovery yielded four practical lessons. First, the development of standard protocols is important to optimize comparisons across effectors and strains. Standardized protocols include bioinformatic methods for identifying Hrp promoters and phylogenomic methods for assigning new *hop* genes to families and subfamilies as well as assays for translocation and other effector phenotypes (Ferreira et al. 2006; Lindeberg et al. 2005; Schechter et al. 2004). Second, ongoing resolution of ambiguities requires a central, frequently updated dataset with

evidence-coding and documentation for all data as well as frequent updates of reference files in GenBank and the TIGR Comprehensive Microbial Resource. Third, a unified nomenclature system for effectors and their functions is essential. Fourth, datasets should be easy to manipulate for regrouping and visual coding in the search for new patterns.

Presently, the two primary tools provided through the website are the Hop Database for *P. syringae* pathovars and a downloadable system for viewing Hrp promoters, effector genes, and other virulence-related features in the genome of each of the sequence *P. syringae* strains using the Artemis Genome Viewer. Table 1 represents a small subset of the main Hop database, which now has over 20 fields of information for nearly 300 Hop/effector/helper proteins from many *P. syringae* strains. The dataset is available for anyone to download and use as a platform for analysis or publication. Associated with the database is a standardized system for naming and adding new Hops (Lindeberg et al. 2005). We anticipate that the Hop database will acquire new data fields to accommodate increasing knowledge of effector function. Also, the use of recently developed Gene Ontology terms for the biological processes, molecular functions, and cellular components of effectors will provide a standard vocabulary to enhance the search for patterns in effector function within plants (Gene Ontology Consortium 2006).

### Conclusions and future challenges.

Bioinformatic analyses and extensive experimental validation in *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A, and *P. syringae* pv. *syringae* B728a have established that the vast majority of T3SS effector genes in *P. syringae* are downstream of high-probability Hrp promoter sequences and possess N-terminal targeting-associated patterns. The effector inventories in each strain contain a complex mix of Hop effectors and helpers, and each strain also carries several inactive effector genes (in families validated in other strains). Although active members of five of the seven helper families appear to be present in each strain, only five of the 46 effector families that are represented in these three strains have an active member in all of the strains. Technological advances that are dramatically reducing the cost of DNA sequencing should enable these bioinformatic methods to efficiently reveal the effector gene inventories of a wider array of strains. A larger collection of analyzed strains can reveal patterns of effector distribution that yield insights into *P. syringae* evolution and host-range determination as well as amino-acid patterns within effector families that predict domains and active sites underlying effector functions in plants (Rohmer et al. 2004).

Many questions still remain regarding the deployment and action of the effector arsenal. How are effectors targeted to the T3SS pathway? How does the pathway apparently sort helper proteins to the apoplast and true effectors to the plant cell cytoplasm? Are the many helpers and effectors delivered in some order? What role do effectors play in the host specificity of *P. syringae* pathovars for different plant species and families? Do the large effector complexes of *P. syringae* strains function more as a coordinated system in defeating multilayered plant defenses or as a redundant assemblage of independent factors interacting with plant populations? The establishment of a suite of resources for discovering and categorizing effectors should enable these and many more questions addressing *P. syringae* effector arsenals to be studied in a systematic way.

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## AUTHOR-RECOMMENDED INTERNET RESOURCE

Pseudomonas-Plant Interaction website: [pseudomonas-syringae.org](http://pseudomonas-syringae.org)