Multiple Approaches to a Complete Inventory of *Pseudomonas syringae* pv. *tomato* DC3000 Type III Secretion System Effector Proteins

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Pseudomonas syringae pv. *tomato* DC3000 is a pathogen of tomato and *Arabidopsis* that translocates virulence effector proteins into host cells via a type III secretion system (T3SS). Many effector-encoding hypersensitive response and pathogenicity (Hrp) outer protein (*hop*) genes have been identified previously in DC3000 using bioinformatic methods based on Hrp promoter sequences and characteristic N-terminal amino acid patterns that are associated with T3SS substrates. To approach completion of the Hop/effector inventory in DC3000, 44 additional candidates were tested by the *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) translocation reporter assay; 10 of the high-probability candidates were confirmed as T3SS substrates. Several previously predicted *hop* genes were tested for their ability to be expressed in an HrpL-dependent manner in culture or to be expressed in planta. The data indicate that DC3000 harbors 53 *hop/lavr* genes and pseudogenes (encoding both injected effectors and T3SS substrates that probably are released to the apoplast); 33 of these genes are likely functional in DC3000, 12 are nonfunctional members of valid Hop families, and 8 are less certain regarding their production at functional levels. Growth of DC3000 in tomato and *Arabidopsis* Col-0 was not impaired by constitutive expression of repaired versions of two *hops* that were disrupted naturally by transposable elements or of *hop* genes that are naturally cryptic. In summary, DC3000 carries a complex mixture of active and inactive *hop* genes, and the *hop* genes in *P. syringae* can be identified efficiently by bioinformatic methods; however, a precise inventory of the subset of Hops that are important in pathogenesis awaits more knowledge based on mutant phenotypes and functions within plants.

Effector proteins translocated into host cells by the type III secretion system (T3SS) are collectively essential to the pathogenicity of many plant- and animal-pathogenic bacteria. Despite their importance, identifying the complete collection of effectors produced by any pathogen has proven difficult. The problems complicating type III effector identification include effector redundancies that can mask mutant phenotypes, the lack of

high-throughput gain-of-function secretion or translocation assays, and the obscure nature of the targeting signals (Sorg et al. 2005). The type III effector inventory of the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 has attracted particular attention. DC3000 is a model pathogen of the important crop tomato and the model plant *Arabidopsis*, and the sequence of the DC3000 genome is known (Buell et al. 2003). As a result of multiple studies from several research groups, more than 40 effector genes have been identified so far in DC3000. These studies also have revealed that most of the encoded effectors have N-terminal targeting-associated patterns that can be useful in predicting effectors. The wealth of information on the type III effectors of this one strain invites completion of the inventory and an assessment of the predictive and functional value of the targeting-associated patterns.

The *P. syringae* T3SS is encoded by hypersensitive response and pathogenicity (*hrp*) and hypersensitive response (HR) and conserved (*hrc*) genes. *P. syringae* type III effectors are designated Hrp outer protein (Hop) and avirulence (Avr) proteins depending on the phenotype by which they were discovered (Lindeberg et al. 2005). All *P. syringae* effectors have a Hop phenotype because they can be secreted or translocated in a T3SS-dependent manner. Some also have an Avr phenotype; when translocated into plant cells carrying a cognate resistance (*R*) gene, either the effector or its activity is recognized, which results in elicitation of the plant HR and resistance. It also should be noted that some Hrp proteins, such as the HrpZ1 and HrpW1 harpins encoded within the Hrp pathogenicity island, are thought to operate primarily in the apoplast as potential extracellular “helpers” in T3SS functions. Some of the newly found Hops also may fall into this category, but we will use the terms effector and Hop broadly here to refer generically to proteins that can be translocated into plant cells by the *P. syringae* T3SS.

Various methods have been used to identify type III effectors in animal and plant pathogens. For example, functional screens have been used to identify effectors in *P. syringae* pv. *maculicola* and *Xanthomonas campestris* pv. *vesicatoria* (Guttman et al. 2002; Roden et al. 2004). Studies of animal pathogen type III effectors include a functional screen for *Chlamydia* proteins with N-terminal fragments capable of directing type III secretion in *Shigella flexneri* (Subtil et al. 2005) and a proteomic analysis of proteins secreted by *Citrobacter rodentium* (Deng et al. 2004). In addition, bioinformatic approaches were used to identify a subset of *Salmonella typhimurium* effectors that have conserved sequences in the first 150 amino acids and *Bordetella* effectors that are associated with chaperones (Miao and Miller 2000; Panina et al. 2005).

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AvrPto1 is a particularly well-studied *P. syringae* pv. *tomato* type III effector. The first *avrPto* gene was cloned from strain JL1065 on the basis of the avirulence phenotype it conferred on *P. syringae* test strains in tomato cultivars carrying the cognate *Pto R* gene (Ronald et al. 1992). Like a typical *P. syringae* effector, AvrPto1 is preceded by an Hrp box promoter and has an N-terminus with an aliphatic amino acid in position 3 or 4, no acidic amino acids in the first 12 residues, and a high percentage of Ser (and Thr) in the first 50 residues (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). AvrPto1 is a useful positive control for genomewide searches for type III effector genes in DC3000, and it has been found in an in planta expression technology (IPET) screen for genes induced during infection (Boch et al. 2002), in a reporter transposon screen for genes activated by the HrpL alternative sigma factor (Fouts et al. 2002), in searches for genes preceded by Hrp box sequences (Fouts et al. 2002; Guttman et al. 2002; Zwiesler-Vollick et al. 2002), in searches for genes predicting type III targeting signals (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002), in a differential fluorescence induction (DFI) screen for genes activated by HrpL (Chang et al. 2005), and in a whole-genome microarray analysis (Ferreira et al. 2006). Each of these studies identified variously overlapping sets of effector candidates in addition to AvrPto1. The differences in these sets relate to ambiguities in Hrp promoter sequences and type III targeting-associated patterns, as well as differences in observed levels of expression and translocation.

Despite the enormous effort to identify effectors in DC3000, several questions remain unanswered. Does DC3000 produce other classes of type III effectors that, unlike AvrPto1, are not associated with Hrp promoters or the recognized targeting-associated patterns? Are all DC3000 type III effectors that pass translocation tests when expressed from vector promoters produced in planta from native promoters? Given the importance of both gene acquisition and gene loss in the evolution of virulence, does the disruption of certain effector genes by transposon insertions allow DC3000 to infect its hosts tomato and *Arabidopsis*? Finally, given the role of DC3000 as a reference strain, what is the “complete” inventory of bona fide type III effector genes in DC3000, including those that are silent, non-functional, or disrupted by transposable elements?

In this article, we address these questions through multiple approaches. First, we tested 44 possible type III effector (Hop) candidates for their ability to be translocated by DC3000 using the quantitative *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) reporter assay. Importantly, many of these candidates were chosen by a variety of criteria other than Hrp promoters and targeting-associated patterns. Second, we used real-time polymerase chain reaction (PCR) and in planta expression technology to assess the expression of several known *hop* effector genes. Third, we assayed the virulence of DC3000 expressing several normally cryptic or disrupted effector genes.

RESULTS

Comprehensive identification of candidate *hop* genes in *P. syringae* pv. *tomato* DC3000.

The criteria depicted in Figure 1 were used to identify 44 additional candidate *hop* genes for T3SS-dependent translocation testing. In general, these criteria addressed two broad categories of candidates: those with a high probability for being effectors but which awaited testing, and lower-probability candidates that could represent novel classes of effectors. Several high-probability candidates were identified on the basis of their association with Hrp boxes as previously defined and then refined based on microarray analysis (Ferreira et al. 2006;

Fouts et al. 2002). The locations of these Hrp promoters and candidate *hop* genes in the DC3000 genome can be visualized using the Artemis genome browser developed by the Sanger Institute. Guidance for the use of the browser and associated data files can be found at the *Pseudomonas*–Plant Interaction website. Other high-probability candidates were identified on the basis of the three major features of *P. syringae* type III targeting-associated patterns. That is, the N-terminal 50 amino acids contain >10% serine; position 3 or 4 contains Ile, Leu, Val, or Pro; and there is no acidic amino acid in the first 12 positions (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). To identify a possible novel class of effectors, we tested several genes that were proximal to known *hop* genes because many *hop* genes are clustered in pathogenicity islands or islets on the DC3000 genome. We also tested several genes encoding proteins that could have activity in eukaryotic signal transduction pathways or with plant cell wall substrates, such as pectate and cellulose. We did not test DC3000 genes that had <100 codons or that had homologs in the nonpathogen *P. putida* KT2440. The candidate genes were cloned by PCR into the pENTR-SD/D-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) and then recombined into Gateway destination vector pCPP3234 (Schechter et al. 2004). The resultant constructs were confirmed by immunoblot analysis to produce the predicted candidate-Cya fusions in DC3000 before being assayed for translocation (data not shown).

Candidate Hop proteins were tested for their ability to be translocated into plant cells by using the Cya reporter.

Candidates were tested with the Cya reporter for their ability to be translocated in a T3SS-dependent manner into *Nicotiana benthamiana* by *P. syringae* pv. *tomato* DC3000 (Schechter et al. 2004).

The translocation of type III effectors carrying C-terminal fusions with Cya can be monitored by the calmodulin-dependent production of cyclic AMP (cAMP) within eukaryote cells

Fig. 1. Strategy for genomewide identification of candidate effector genes in *Pseudomonas syringae* pv. *tomato* DC3000. The DC3000 genome was scanned for genes that possess one or more of the four features listed on the left side of the figure and described further in the text. From this list, genes described on the right side of the figure were excluded from further investigation. Effector candidates encoded by the 44 remaining genes were tested for translocation into plant cells. Hrp = hypersensitive response and pathogenicity, T3SS = type III secretion system, and Hop = Hrp outer protein.

(Casper-Lindley et al. 2002; Sory and Cornelis 1994). The Cya translocation reporter assay yields quantitative data on translocation efficiencies, and the resulting comparative data complements other effector surveys that used the AvrRpt2₍₈₁₋₂₅₅₎ translocation reporter (Chang et al. 2005; Vinatzer et al. 2005). Candidate genes were expressed from the *tac* promoter in pCPP3234 to provide adequate expression for definitive translocation tests and to permit comparison of the translocation efficiencies of equally expressed proteins. It also is important to note that Cya was fused to the full-length Hop candidate in most cases. However, in certain cases, the candidates were so large (>900 amino acids) that we had to make fusions with C-terminally truncated versions of these proteins (Tables 1 and 2).

Most of the high-probability Hop candidates were confirmed to be translocated in a T3SS-dependent manner.

Ten of the high-probability candidates were translocated into plant cells (Fig. 2). Table 1 indicates whether they have any homologs or domains predictive of function, as well as the status of each Hop regarding Hrp regulation and type III targeting-associated patterns. The translocation of PSPTO0907 was weak compared with the others, but it was consistently higher with DC3000 than with the $\Delta hrpL$ mutant CUCPB5114. While this work was in preparation, several of these Hops (HopR1, HopY1, HopAH1, HopAH2-1, HopAII, and HopT1-2) were shown to travel the Hrp T3SS by other methods (Chang et al. 2005; Guo et al. 2005; Vinatzer et al. 2005). However, four of the confirmed Hops in Table 1 had not

been tested before. PSPTO4592 encodes a member of the HopO family and, thus, it already had been named HopO1-3' (Lindeberg et al. 2005). Although the C-terminus of HopO1-3' is truncated due to a premature stop codon, our results show that the N-terminal region of this protein allows the delivery of the Cya reporter in plant cells. PSPTO3293 encodes a protein closely related to HopAH2. Therefore, we renamed PSPTO3292 HopAH2-1 and will call PSPTO3293 HopAH2-2. PSPTO0474 had not been identified previously as a T3SS substrate, and we named it HopAS1'. HopAS1' represents a 279-amino acid frameshift truncation of a much larger effector that is activated by HrpL and translocated by *P. syringae* pv. *phaseolicola* 1448A (Vencato et al. 2006). PSPTO0907 also is translocated by DC3000; however, because it is not produced in an HrpL-dependent manner, we cannot designate it as a Hop unless one or more compliant Hop homologs are found in other strains of *P. syringae* (Lindeberg et al. 2005). Interestingly, 3 of the 10 Hop proteins validated as T3SS substrates in this study contained only two of three primary patterns used to predict T3SS substrates (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004), and 2 contained only one of the patterns. As shown below, the *hop* genes encoding these proteins were not activated by HrpL.

Three DC3000 Hop candidates with functional hop homologs were found not to be translocated.

The candidates presented in Table 2 were judged as not being translocated because they did not allow the accumulation

Table 1. Features of Hop proteins confirmed to be translocated into plant cells by the DC3000 type III secretion system in this study

Locus ^s	Gene ^t	Homologs or domains (BLAST; GenBank) ^u	HrpL ^v	Translocation signals		
				≥10% S ^w	ILVP ^x	No DE ^y
0061	<i>hopY1</i>	DC3000 HrpA1 (6e-8; AAO54903)	+	+	+	+
0474	<i>hopAS1'</i>	<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> 1448a HopAS1 (3e-133; AAZ37064)	±	–	+	+
0883	<i>hopR1</i> ^z	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448a HopR1 (0; AAZ37024)	+	+	+	+
		<i>Xanthomonas campestris</i> pv. <i>campestris</i> XCC1089 (0; AAM40388)				
		<i>Ralstonia solanacearum</i> GMI1000 RSp1281 (1e-72; CAD18432)				
0905	<i>hopAH1</i>	Cellulase-glycosyl hydrolase family 5 (1e-4; Pfam 00150)	–	+	–	+
		<i>P. syringae</i> pv. <i>syringae</i> B728a HopAH1 (3e-145; AAY35837)				
		DC3000 HopAH2-1 (1e-96; AAO56770)				
		<i>P. syringae</i> pv. <i>phaseolicola</i> 1448a HopAH2 (8e-86; AAZ34780)				
		DC3000 HopAH2-2 (3e-83; AAO56771)				
0906	<i>hopAII</i>	<i>P. syringae</i> pv. <i>syringae</i> B728a HopAII (3e-93; CP000075)	–	–	+	+
		<i>Shigella flexneri</i> M90T OspF (2e-35; CAC05773)				
		<i>Chromobacterium violaceum</i> ATCC12472 CV1308 (1e-34; AAQ58983)				
		<i>Salmonella typhimurium</i> LT2 SpvC (2e-33; AAL23529)				
0907	NA	None	–	+	+	+
3292	<i>hopAH2-1</i>	Cellulase-glycosyl hydrolase family 5 (7e-3; Pfam 00150)	–	+	+	+
		<i>P. syringae</i> pv. <i>phaseolicola</i> 1448a HopAH2 (2e-140; AAZ34780)				
		DC3000 HopAH2-2 (4e-136; AAO56771)				
		<i>P. syringae</i> pv. <i>syringae</i> B728a HopAH1 (3e-123; AAY35837)				
		DC3000 HopAH1 (2e-122; AAO54439)				
3293	<i>hopAH2-2</i>	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448a HopAH2 (3e-147; AAZ34780)	–	–	+	–
		DC3000 HopAH2-1 (2e-112; AAO56770)				
		DC3000 HopAH1 (2e-87; AAO54439)				
		<i>P. syringae</i> pv. <i>syringae</i> B728a HopAH1 (2e-86; AAY35837)				
4592	<i>hopO1-3'</i>	DC3000 HopO1-2 (3e-46; AAO58040)	–	+	+	+
		DC3000 HopO1-1 (2e-26; AAO59043)				
4593	<i>hopT1-2</i>	DC3000 HopT1-1 (2e-155; AAO59044)	–	+	–	–
		<i>P. luminescens laumondii</i> PLU3136 (1e-26; CAE15510)				

^s PSPTO open reading frame number.

^t Gene names are based on the nomenclature described by Lindeberg and associates (2005); NA = no Hop designation can be given because although PSPTO0907 is translocated, its expression is not HrpL-dependent and it is not a member of a Hop family with functional representatives (Lindeberg et al. 2005).

^u Homologs or similar domains (BLAST *e* value; GenBank accession number).

^v Regulation by HrpL, as determined in (Ferreira et al. 2006) or this work; ± indicates very weak regulation.

^w At least 10% Ser residues within the first 50 amino acids of the protein is indicated by +.

^x An Ile, Leu, Val, or Pro located in the third or fourth residue of the protein is indicated by +.

^y No Asp or Glu residues within the first 12 amino acids of the protein is indicated by +.

^z The first 201 amino acids of HopR1 were fused to *Bordetella pertussis* adenylate cyclase due to the large size of this protein (1,957 amino acids).

of more than 5 pmol cAMP per microgram of protein in plants (data not shown). Three candidates, HopT2, HopQ1-2, and HopAC1, show similarity to confirmed Hops, HopT1-1, HopQ1-1, and HopB1, respectively. It is important to note that each of these inactive homologs is not similar to its respective confirmed Hop in the N-terminal region that is required for targeting to the T3SS. In fact, the *hopAC1* gene in DC3000 appears to be disrupted by a transposable element, because a much larger intact version of this gene is present in *P. syringae* pv. *syringae* B728a (Greenberg and Vinatzer 2003). However, the full-length HopAC1 from B728a also is not translocated into plant cells (B. Vinatzer, *personal communication*).

Genes encoding several of the newly confirmed Hop proteins in DC3000 are not activated by HrpL.

Eight of the genes encoding proteins shown to be translocated in this study were not observed to be regulated by HrpL in a whole-genome microarray analysis (Ferreira et al. 2006). We performed real-time PCR analyses of these genes in wild-type DC3000 and Δ *hrpL* mutant UNL134-1 grown in Hrp-inducing minimal medium to further test whether these genes

are HrpL responsive (Fig. 3). Two of these genes, *hopR1* and *hopAS1'*, are located downstream from Hrp boxes. We found that *hopR1* expression was activated by HrpL, but its induction was weak compared with that of *hrcS*, a known HrpL-regulated gene (Fig. 3). The *hopAS1'* gene also was very weakly regulated by HrpL. The *hrpL* deletion did not affect the expression of the *hopAH1*, *hopO1-3'*, *hopT1-2*, or PSPTO0907 genes. This is not surprising, because these genes appear to be located in operons that are disrupted by transposable elements. The *hopAH2-1* and *hopAH2-2* genes, which are not located downstream from *hrp* boxes, also were not found to be regulated by HrpL.

Genes encoding several of the newly confirmed Hop proteins do not appear to be expressed by DC3000 in planta.

We next asked whether selected genes encoding newly confirmed Hop proteins that were activated by HrpL only weakly or not at all were nevertheless expressed in planta. We employed a directed version of the IPET (in vivo expression technology) strategy, which is based on the finding that a T3SS-deficient

Table 2. Hop candidates found not to be translocated into plant cells by the DC3000 type III secretion system in this study

Locus (gene) ^a	Homologs or domains (BLAST; GenBank) ^b	HrpL ^c	Translocation signals		
			≥10% S ^t	ILVP ^u	No DE ^v
0400	Glycosyl hydrolase family 18 (3e-11; Pfam 00704)	— ^w	—	+	+
0471	Putative nucleotidyltransferase DUF294 (3e-63; Pfam 03445)	—	+	+	+
0473 ^x	<i>Xanthomonas axonopodis</i> citri 306 XAC2009 (1e-6; AAM36871) ^y	—	—	+	+
0475	Conserved hypothetical protein of unknown function ^z	—	+	+	+
0848	Conserved hypothetical protein of unknown function ^z	—	+	—	+
0849	Conserved hypothetical protein of unknown function ^z	—	+	+	+
0869	Conserved hypothetical protein of unknown function ^z	— ^w	—	+	+
1409	Conserved hypothetical protein of unknown function ^z	— ^w	—	—	+
1657	<i>Pseudomonas syringae</i> tomato DC3000 PSPTO0021 (2e-161; AAO53577)	—	+	—	—
2870	Conserved hypothetical protein of unknown function ^z	—	—	—	—
2871 ^x	Conserved hypothetical protein of unknown function ^z	—	—	+	—
2873	Conserved hypothetical protein of unknown function ^z	—	—	+	+
2874	<i>P. aeruginosa</i> PAK PpkA (7e-99; AAD03499)	—	—	+	+
2877	Conserved hypothetical protein of unknown function ^z	—	—	—	+
2878	Conserved hypothetical protein of unknown function ^z	—	+	+	+
2880	Von Willebrand factor type A domain (1e-8; cd01456)	—	+	+	—
3120	Cof hydrolases of the HAD superfamily (4e-47; COG0561)	— ^w	+	+	—
3483	<i>P. fluorescens</i> PfO-1 Pfl0152 (3e-49; ABA71896)	— ^w	+	+	—
3534	Glycosyl hydrolase family 5 (8e-7; Pfam00150)	—	—	—	+
4340 ⁱ	<i>P. luminescens</i> insecticidal toxin TccC (3e-126; AAO17196)	—	+	+	+
4342 ^x	<i>P. luminescens</i> insecticidal toxin TccB (9e-152; AAL18472)	—	—	—	—
4343 ^x	<i>P. luminescens</i> insecticidal toxin TcdB (0; AAO17202)	—	+	—	—
4344 ^x	<i>P. luminescens</i> insecticidal toxin TccC (1e-118; AAO17204)	—	—	—	—
4590 (<i>hopT2</i>)	DC3000 HopT1-2 (5e-25; AAO58039)	—	+	—	+
4699 ^x	Non-ribosomal peptide synthetase module (1e-155; COG1020)	— ^w	+	+	+
4732 (<i>hopQ1-2</i>)	DC3000 HopQ1 (2e-56; AAO54411)	+	+	+	—
4733	<i>Ralstonia metallidurans</i> CH34 RMe0230 (5e-7; CAI11198)	— ^w	—	+	+
4956	Conserved hypothetical protein of unknown function ^z	— ^w	+	—	+
4996 (<i>hopAC1</i>) ^x	DC3000 HopB1 (6e-16; AAO54928)	—	+	—	—
5355	<i>P. syringae</i> pv. <i>phaseolicola</i> PSPPH4936 (8e-65; AAZ33056)	—	—	+	—
5416	Protein kinase domain (8e-31; Pfam 00069)	—	—	+	—
5417	Serine/threonine phosphatases family 2C (2e-34; cd00143)	—	—	+	+
5432	Conserved hypothetical protein of unknown function ^z	—	+	+	+
B0005	Phosphoesterase family (5e-44; Pfam 04185)	+	+	—	—

^a PSPTO open reading frame (ORF) number (gene name).

^b Homologs or similar domains (BLAST *e* value; GenBank accession number).

^c Regulation by HrpL, as determined by Ferreira and associates (2006) or this work; ± indicates very weak HrpL-dependent expression.

^d At least 10% serine residues within the first 50 amino acids of the protein is indicated by +.

^e An Ile, Leu, Val, or Pro located in the third or fourth residue of the protein is indicated by +.

^f No Asp or Glu residues within the first 12 amino acids of the protein is indicated by +.

^g Gene is downstream from an *hrp* box but no regulation by HrpL was detectable (Ferreira et al. 2006).

^h Due to the large size of these proteins, the following number of N-terminal amino acids were fused to *Bordetella pertussis* adenylate cyclase: 207 of PSPTO0473, 214 of PSPTO2871, 200 of PSPTO4340, 103 of PSPTO4342, 100 of PSPTO4343, 101 of PSPTO4344, 204 of PSPTO4699, and 400 of PSPTO4996.

ⁱ This ORF encodes a protein that is homologous to the C-terminal region of HopAS1 in *P. syringae* pv. *phaseolicola* 1448a.

^j ORFs that were highly similar to more than two bacterial proteins in GenBank.

Fig. 2. Translocation of candidate effectors based on increased cyclic AMP (cAMP) levels in *Nicotiana benthamiana* following inoculation with DC3000 strains expressing calmodulin-dependent adenylate cyclase (Cya) hybrid proteins. *N. benthamiana* leaves were inoculated with either DC3000 or CUCPB5114 (DC3000 $\Delta hrpK$ -*hrpR*:: Ω Cm) bacteria expressing the indicated Cya hybrid proteins from the vector *tac* promoter. The proteins that were N-terminally fused to Cya are denoted by both their open reading frame number and hypersensitive response and pathogenicity (Hrp) outer protein (Hop) name. Leaf samples were collected with a 0.8-cm-diameter cork borer 7 h post inoculation. cAMP and protein levels were quantified in duplicate and averaged for each sample. The average amount of cAMP per microgram of protein for each sample is indicated above the appropriate bar in the graph, and standard deviations are indicated by error bars. Repeated experiments yielded similar relative levels of cAMP production for each of the strains tested.

Fig. 3. Analysis of HrpL regulation of hypersensitive response and pathogenicity (Hrp) outer protein (*hop*) gene expression in *Pseudomonas syringae* pv. tomato DC3000 by real-time polymerase chain reaction (PCR). Quantitative SYBR green-based real-time PCR was performed on cDNA prepared from either DC3000 or UNL134-1 (DC3000 $\Delta hrpL$) grown in *hrp*-derepressing fructose minimal medium, as described elsewhere (Vencato et al. 2006). The relative fold induction of each gene by HrpL was determined by the standard curve method after normalization to either *gap1* or *gyrA*, two housekeeping genes that served as endogenous controls. The numbers reported are the average and standard deviation of values obtained by two different normalization methods in three separate experiments, as described (Vencato et al. 2006).

DC3000 *hrcC* mutant is unable to cause disease or elicit the HR unless HrcC production is restored through a *hrcC* gene in trans expressed from a test promoter that is active in planta (Boch et al. 2002). DNA fragments containing the *hopR1*, *hopAS1'*, *hopAH2-1*, and *hopAH2-2* promoters were cloned into the kanamycin-resistant pIPET plasmid upstream of the promoterless *hrcC* gene, and these plasmids were transferred into TLR1 (DC3000 $\Delta hrcC::Sp^R$). Because pIPET cannot replicate in *P. syringae*, the plasmid must recombine into the chromosome at sites homologous to the cloned promoter fragment to confer kanamycin resistance. If these promoters are active in planta, they will drive expression of the promoterless *hrcC* gene in pIPET, which would complement the deletion of the native *hrcC* in TLR1. As a negative control, plants were inoculated with JB201, a TLR1 strain that contains pIPET inserted downstream from the reverse orientation of the *avrPto1* promoter. As a positive control, plants were inoculated with JB200, a TLR1 strain that contains pIPET inserted in the forward orientation downstream from the *avrPto1* promoter. We observed elicitation of the HR in each of 12 tests involving JB200 and with CUCPB5431, which contains pIPET downstream of the *hopR1* promoter. However, the HR produced by CUCPB5431 was delayed by approximately 24 h compared with JB200, which may be due to the weak activation of *hopR1* by HrpL. We did not observe an HR in repeated tests involving the *hopAS'*, *hopAH2-1*, and *hopAH2-2* promoters.

Growth in tomato and *Arabidopsis* of *P. syringae* pv. *tomato* DC3000 is not impaired by constitutively expressing normally cryptic or disrupted *hop* genes.

It has been suggested that diverse combinations of effectors in different *P. syringae* pathovars may be responsible for the di-

verse host ranges of these strains (Greenberg and Vinatzer 2003). The presence in DC3000 of several cryptic or disrupted *hop* genes raises the possibility that, if these genes were functional, they would cause DC3000 to become avirulent in hosts tomato and *Arabidopsis*. To test this possibility, we constructed derivatives of DC3000 and $\Delta hrp/hrc$ mutant CUCPB5114 carrying CPP3234 expressing Hop-Cya fusions of the test effectors from the vector *tac* promoter. Two of the effector genes that were tested, *hopAG::ISPssy* and *hopS1::ISPssy*, were repaired using PCR to remove disrupting transposon sequences; and five others, *hopAH1*, *hopA11*, *hopO1-3'*, *hopT1-2*, and PSPTO0907 appeared unlikely to be expressed in planta. Tomato cv. Money-maker plants were inoculated by dipping with DC3000 strains that contained plasmids expressing various Hop-Cya fusion proteins that had been shown to be delivered into plants (data not shown; Fig. 2). As a control, plants also were infected with DC3000 expressing AvrPto-Cya, which does not cause the HR in Money-maker tomato. None of the Hop-Cya fusion proteins reduced the growth of DC3000 in tomato plants at either 2 or 5 days post inoculation (Fig. 4). Similar results were obtained in an experiment using *Arabidopsis* Col-0 plants as the host (data not shown). A slight increase in the growth of DC3000 expressing HopAH1 can be seen in Figure 4; however, the increase is not statistically significant based on a *t* test (where equal variances were not assumed) and on a repeated measures analysis of variance test. Furthermore, there was no increased growth of this strain in *Arabidopsis*. Bacterial colonies that were recovered from plants 3 days post infection were patched on selective medium to examine whether they maintained the plasmid expressing the Hop-Cya fusion protein. For each strain, 80 to 100% of the colonies had maintained the plasmid. In summary, none of the test effectors caused DC3000 to become avirulent on hosts tomato and *Arabidopsis*.

Fig. 4. Growth of *Pseudomonas syringae* pv. *tomato* DC3000 strains expressing hypersensitive response and pathogenicity (Hrp) outer protein (Hop) calmodulin-dependent adenylate cyclase (Cya) fusion proteins in tomato. Tomato (cv. Money-maker) plants were inoculated by dipping in CUCPB5114 (DC3000 $\Delta hrpK-hrpR::\Omega Cm$), wild-type DC3000, or DC3000 strains at 5×10^6 CFU ml⁻¹ expressing the indicated Hop-Cya fusion proteins. The number of bacteria per milligram of leaf tissue was determined as described in the Materials and Methods at the indicated number of days post infection. Each value is the mean of four data points, and error bars indicate the standard deviations.

DISCUSSION

We have used multiple approaches aimed at determining the complete inventory of effector genes in *P. syringae* pv. *tomato* DC3000. These approaches represent the culmination of a chain of studies in which we used bioinformatic and experimental techniques to find and verify type III effectors in this model pathogen. Below, we will discuss several aspects of the DC3000 effector inventory: i) the strengths and weaknesses of different approaches to effector identification in *P. syringae*, ii) the existence of nonfunctional effectors, and iii) the current status of the inventory. A summary of the current DC3000 effector inventory, with the features discussed below highlighted, is presented in Table 3.

Evaluation of the candidate gene approach for identifying type III effectors in *P. syringae* pv. *tomato* DC3000.

Our chain of studies has used a three-step method to identify type III effectors in DC3000. First, Hrp promoter sequences with a high probability of functionality were identified by a hidden Markov model and other criteria (Ferreira et al. 2006; Fouts

et al. 2002). Second, candidate effector genes downstream of such promoters were predicted based on targeting-associated amino acid patterns (Petnicki-Ocwieja et al. 2002). Third, the ability of candidate genes to produce type III substrates was tested by translocation or secretion assays employing a vector *tac* promoter whose strength in pseudomonads approximates that of well-expressed Hrp promoters (Schechter et al. 2004). The present study completes the testing of candidates generated by the first two steps. Identification of the effector inventory in DC3000 also has been advanced by a candidate gene approach similarly taken by J. Greenberg, D. Guttman, B. Vintazer, and their colleagues (Greenberg and Vinatzer 2003; Guttman et al. 2002; Vinatzer et al. 2005). Importantly, the success of these bioinformatics-based approaches now can be gauged by comparison with the recent near-saturation DFI screen and translocation tests of Chang and associates (2005) (Table 3).

The DFI-based study identified 25 DC3000 proteins that, when fused to $\Delta 79$ AvrRpt2, reliably elicited an RPS2-dependent HR in *Arabidopsis* that is indicative of translocation. Twenty of these proteins were identified through the DFI screen itself, but all of them had been reported previously in bioinformatic-driven studies (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002;

Table 3. Inventory of genes encoding Hop/effector proteins in *Pseudomonas syringae* pv. *tomato* DC3000

Locus [†]	Name [‡]	Expression [‡]	T3SS [‡]	Secretion/translocation		Phenotype observed [‡]
				Heterologous promoter ^x	Native promoter ^y	
0044	<i>hopK1</i>	D, M	1,2,3; H	ab	+	
0061	<i>hopY1</i>	D, M, T	1,2,3; H	cd	+	
0474	<i>hopAS1'</i>	M	2,3; H	c	NT	
0501	<i>hopU1</i>	Alfano, unpublished	1,2,3; H	a	–	Alfano, unpublished
0502	<i>hopF2</i>	D, M	1,2,3; C; H	ef	+	Jamir et al. 2004
0588	<i>hopH1</i>	D, M	1,2,3	a	+	
0589	<i>hopC1</i>	D, M, T	1,2,3; H	ab	+	
0852	<i>hopAJ1</i>	D, M, T	1,2,3; H	NT	–	
0876	<i>hopD1</i>	D, M	1,2,3; H	a	+	
0877	<i>hopQ1-1</i>	D, M	1,2,3	bd	+	
0883	<i>hopR1</i>	D, M, T	1,2,3	cd	NT	
^0901	<i>hopAG::ISPssy</i>	D	1,2,3; H	b	–	
^0905	<i>hopAH1*</i>	NE	1,3; H	cd	NT	
^0906	<i>hopAI1*</i>	NE	2,3	cd	NT	
^0907	–*	NE	1,2,3	c	NT	
1022	<i>hopAMI-1</i>	D, M, I	1,3; H	NT	+	Jamir et al. 2004
^1179	<i>hopJ1</i>	–, no Hrp box	1,2,3	N-terminal fragment	–	
1370	<i>hopN1</i>	D, M, T, I	1,2,3; C	h	+	
1372	<i>hopAA1-1</i>	D, M, T	1,2,3; H	i	+	Badel et al. 2002
1373	<i>hrpW1</i>	D, M, T	1,2,3; H	g	+	

(continued on next page)

[†] PSPTO open reading frame (ORF) locus number. Genes are presented in the order of their PSPTO number along the DC3000 chromosome, with the four plasmid-borne effector genes (PSPTO numbers preceded with letter A) being presented last. PSPTO numbers greater than 5615 were added after the initial annotation and do not correlate with relative position in the genome.

[‡] Genes in putative operons where an upstream ORF is disrupted by a transposable element are marked with an asterisk (*). Although the previously assigned *hop* names are shown for PSPTO1179, PSPTO2872, and PSPTO4996, these genes are not regulated by HrpL and are not members of any validated effector family; therefore, they do not meet the current criteria for Hops (Lindeberg et al. 2005). Bold type indicates that the Hop is likely active in DC3000. A caret (^) at the beginning of the row indicates that the Hop is not active in DC3000, and no special marking indicates that the functional status of the Hop is uncertain.

^y Genes found to be regulated by HrpL or expressed in planta. The letters in this column indicate that the gene was found to be expressed in the following assays: D, differential fluorescence induction (DFI) (Chang et al. 2005); M, microarray or real-time polymerase chain reaction (RT-PCR) (Ferreira et al. 2006); I, in planta expression technology (IPET) (Boch et al. 2002); R, RT-PCR (Guo et al. 2005); T, miniTn5gus insertion (Fouts et al. 2002); and NE indicates that the gene was not found in any of the above studies. Note that the presence of two identical copies of *hopAMI* complicates microarray analysis, and *hopAN1* expression is activated by HrpL despite lacking an upstream Hrp box (Boch et al. 2002).

^x Type III secretion system (T3SS) bioinformatic evidence for classification as an effector. The numbers and letters in this column represent the following characteristics: 1, >10% Ser within the first 50 amino acids; 2, an Ile, Leu, Val, or Pro at position 3 or 4; 3 no Asp or Glu within the first 12 codons; H, sequence similarity to other Hops; C, interaction with T3SS chaperones.

^x Evidence for secretion or translocation when the protein is heterologously expressed. Experiments reporting secretion or translocation of the DC3000 protein are reported in: a, Petnicki-Ocwieja et al. 2002; b, Schechter et al. 2004; c, this work; d, Vinatzer et al. 2005; e, Zwiesler-Vollick et al. 2002; f, Shan et al. 2004; g, A. Collmer, unpublished data; h, López-Solanilla et al. 2004; i, Badel et al. 2002; j, Badel et al. 2003; k, J. R. Alfano, unpublished data; l, van Dijk et al. 1999; m, Badel et al. 2006; n, Guo et al. 2005; o, Kabisch et al. 2005; p, Petnicki-Ocwieja et al. 2005.

^y Evidence for translocation when the gene is expressed from its native promoter as reported by Chang and associates (2005): +, protein is translocated; –, protein is not translocated; ±, protein was inconsistently translocated; NT, not tested. Tests for HopS1' and HopT1-1 are from Vinatzer and associates (2005).

^z Mutant phenotype in planta observed.

Schechter et al. 2004). A notable feature of the DFI study is that the follow-up translocation tests employed candidate effector genes expressed in DC3000 from their native promoter (in trans on a stable, multicopy plasmid). In addition to the 25 effectors that were reliably translocated by this approach, there were 6 proteins involving fusions with partial-length test proteins or which yielded inconsistent results, but which were cautiously scored as effectors. In essence, the DFI and native promoter translocation tests identified what appear to be the 25 most strongly expressed effector genes in DC3000. A whole-genome microarray and bioinformatic analysis of HrpL-responsive DC3000 genes also highlights this set of strongly expressed effectors (Ferreira et al. 2006). In summary, the most strongly expressed effector genes can be found through their Hrp promoters by reporter transposons (Fouts et al. 2002), DFI (Chang et al. 2005), microarray analysis (Ferreira et al. 2006), or more directly and efficiently by bioinformatic identification of such promoters in the genome sequence (Fouts et al. 2002; Guttman et al. 2002; Zwiesler-Vollick et al. 2002).

The analysis by Chang and associates (2005) also confirmed the utility of targeting-associated patterns in predicting which genes downstream of Hrp promoters encode T3SS substrates.

That is, 26 of the 31 confirmed effectors possessed all three of the primary patterns predicting T3SS substrates (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). The five exceptions were HopV1 (possessing none of the targeting-associated patterns) and HopP1, HopM1, HopAM1-1, and HopAM1-2 (all lacking the aliphatic amino acid signal pattern). However, HopAM1-1 and HopAM1-2 (which are identical proteins in DC3000) contain a hydrophobic amino acid (A) in position 3, which appears in a few other Hrp-secreted proteins (Petnicki-Ocwieja et al. 2002). Importantly, the five exceptions all possessed additional features that would keep them on a list of candidates worth testing in a bioinformatic analysis: the *hopV1* and *hopM1* genes are linked with obvious chaperone genes, HopP1 has a strikingly high Ser bias in its N-terminal region, and HopAM1-1 and HopAM1-2 are both homologs of the known effector AvrPpiB1. In summary, the data from Chang and associates (2005) validates the bioinformatic approach for identification of candidate effectors based on the presence of Hrp promoters and T3SS targeting-associated patterns.

It is noteworthy that targeting-associated patterns also are found in flagellar secretion substrates and in a subset of T3SS effectors in other plant and animal pathogens. For example, the

Table 3. (continued from preceding page)

Locus [†]	Name [‡]	Expression [‡]	TTSS [‡]	Secretion/translocation		Phenotype observed [‡]
				Heterologous promoter [‡]	Native promoter [‡]	
1375	<i>hopM1</i>	D, M, I	1,3; C	j	+	Badel et al. 2003
1377	<i>avrE1</i>	D	1,2,3; H; C	m	±	Badel et al. 2006
1382	<i>hrpZ1</i>	D, M	1,2,3; H	g	+	
1406	<i>hopB1</i>	D, M	1,2,3; H	p	+	
1568	<i>hopAF1</i>	D, M	1,2,3; H	a	+	
2678	<i>hopP1</i>	D, M	1,2,3;	d	+	Collmer, unpublished
^1179	<i>hopJ1</i>	–, no Hrp box	1,2,3	N-terminal fragment	–	
1370	<i>hopN1</i>	D, M, T, I	1,2,3; C	h	+	
1372	<i>hopAA1-1</i>	D, M, T	1,2,3; H	i	+	Badel et al. 2002
1373	<i>hrpW1</i>	D, M, T	1,2,3; H	g	+	
1375	<i>hopM1</i>	D, M, I	1,3; C	j	+	Badel et al. 2003
1377	<i>avrE1</i>	D	1,2,3; H; C	m	±	Badel et al. 2006
1382	<i>hrpZ1</i>	D, M	1,2,3; H	g	+	
1406	<i>hopB1</i>	D, M	1,2,3; H	p	+	
1568	<i>hopAF1</i>	D, M	1,2,3; H	a	+	
2678	<i>hopP1</i>	D, M	1,2,3;	d	+	Collmer, unpublished
^2872	<i>hopL1</i>	–, no Hrp box	2,3	a	–	
3087	<i>avrPtoB</i>	D, M, T, I	1,2,3; H	e	+	Lin and Martin 2005
^3292	<i>hopAH2-1</i>	NE	1,2,3; H	cd	NT	
^3293	<i>hopAH2-2</i>	NE	2; H	c	NT	
4001	<i>avrPto1</i>	D, M, T, I	1,2,3; H	l	+	Lin and Martin 2005
4101	<i>hopAK1</i>	D, M, T, I	2,3; H	g	–	Collmer, unpublished
4331	<i>hopE1</i>	D, M	1,2,3	ae	+	Alfano, unpublished
4588	<i>hopS2</i>	D, R	1,3; H; C	bn	–	
^4590	<i>hopT2*</i>	NE	1,3; H	c	NT	
^4592	<i>hopO1-3' *</i>	NE	1,2,3; H	c	NT	
4593	<i>hopT1-2*</i>	R	1; H	cdn	–	
4594	<i>hopO1-2*</i>	R	1,2,3; H; C	kn	±	
^4597	<i>hopS1::ISPssy</i>	D, M, I, R	1,2,3; H; C	bno	+	
4691	<i>hopAD1</i>	D	1,2,3	a	–	
4703	<i>hopAQ1</i>	D	1,2,3	d	–	
4718	<i>hopAA1-2</i>	I, M	1,2,3; H	g	+	Badel et al. 2002
4720	<i>hopV1</i>	M	C	b	+	
4722	<i>hopAO1</i>	D, M, I	1,2,3; H	a	NT	Espinosa et al. 2003
^4724	<i>hopD::IS52</i>	D	1,2,3; H	NT	NT	...
4727	<i>hopG1</i>	D, M	1,2,3	a	±	Jamir et al. 2004
^5623	<i>hopH::ISPssy4</i>	NE	2,3; H	NT	NT	...
^4732	<i>hopQ1-2</i>	M	1,2; H	NT	NT	...
4776	<i>hopI1</i>	D, M, T	1,2,3; H	NT	+	...
^4996	<i>hopAC'</i>	NE	1; H	NT	NT	...
5061	<i>hopAN1</i>	I	1,2,3	NT	NT	...
5354	<i>hopA1</i>	D, M, T	1,2,3; H; C	NT	+	...
A0005	<i>hopAM1-2</i>	M	1,3; H	g	NT	...
A0012	<i>hopX1</i>	M, T	1,2,3; H	b	±	Jamir et al. 2004
A0018	<i>hopO1-1</i>	M	1,2,3; H; C	an	+	...
A0019	<i>hopT1-1</i>	M	1,2,3; H	b	+	...

Yersinia effector YopE possesses the three major patterns, including an unusually high Ser content of 28% in the first 50 residues. However, other *Yersinia* effectors differ. For example, YopQ has an Asp in position 5 and a Ser content of only 6% in the first 50 residues, and YopT and YopH have an Asp within the first 7 residues. Observations such as this raised the possibility that there could be another class of effectors with quite different N-terminal amino acid patterns. However, we found no evidence for such a class despite translocation assays of the products of many candidate genes that were either downstream of Hrp promoters, linked with known effector genes, or that predicted properties that can be associated with eukaryotic functions. Thus, our analysis suggests that searching *P. syringae* genomes for open reading frames (ORFs) that are downstream of Hrp promoters and that possess the targeting-associated amino acid patterns (or are linked to candidate chaperone genes) will yield virtually all of the effectors in that strain. Although these patterns have confirmed predictive value in *P. syringae*, it is important to note that their function remains to be tested.

The analysis of Chang and associates (2005) also revealed 10 candidates that did *not* deliver $\Delta 79\text{AvrRpt2}$ into plant cells despite possession of all of the targeting-associated patterns. This raises the concern that analysis of HrpL-regulated genes for targeting-associated patterns yields false positives. There are several reasons why we think that this is not the case. At least five of these predicted proteins are candidate helpers (proteins such as harpins that travel the T3SS but, unlike true effectors, are targeted to the apoplast rather than the host cytoplasm) (Collmer et al. 2002). These proteins would be expected to inefficiently deliver $\Delta 79\text{AvrRpt2}$ to plant cells. Of the remaining four proteins, HopAQ1 is otherwise untested, but HopS2, HopAD1, and HopT1-1 had been reported previously to be secreted or translocated by the T3SS when constitutively expressed from vector promoters (Petnicki-Ocwieja et al. 2002; Schechter et al. 2004). We consider these to be true effectors, although we cannot say that they are active in DC3000 pathogenesis.

Several potential limitations in the AvrRpt2 delivery test are worth noting. Some effectors may suppress HR development and, thereby, interfere with the assay (Abramovitch et al. 2003; Jackson et al. 1999; Jamir et al. 2004). For example, HopX1 and HopG1 are suppressors of cell death that have mutant phenotypes (enhanced HR in nonhost tobacco) but were rated as producing only "inconsistent translocation results" by Chang and associates (2005). It also is possible that some effectors may specifically block AvrRpt2 action by protecting RIN4 (Axtell and Staskawicz 2003; Mackey et al. 2003). A more general problem is sensitivity. AvrRpt2 has been reported to produce a weaker HR than other effectors, such as AvrRpm1 and AvrB (Leister et al. 1996; Reuber and Ausubel 1996; Ritter and Dangl 1996). More importantly, we presently have no idea what levels are needed for the virulence functions of any effector.

We can expect the dynamics of effector genetics in *P. syringae* to involve not only the obvious events of horizontal acquisition, deletion, and disruption by mobile genetic elements but also more subtle changes affecting transcription, translation, stability, and effector delivery. Furthermore, as discussed below, loss of effector function actually may contribute to virulence. Therefore, we think that it is important to identify all effectors (whether active or not) in reference strains such as DC3000 and to analyze separately each stage in the deployment of these effectors. Quantitative Cya translocation reporter assays employing effectors expressed from a reference promoter such as the *tac* promoter are an important part of this analysis, and these standardized translocation assays usefully complement

microarray and real-time PCR analyses of the relative expression of the respective effector genes.

Analysis of nonfunctional effectors.

Effectors can betray *P. syringae* strains to the *R* gene surveillance system, and they can interfere with each other's action. Furthermore, it is likely that *P. syringae* strains can readily acquire new effectors because many effector genes are associated with mobile genetic elements, recombination hot spots, and other factors associated with horizontal acquisition (Alfano et al. 2000; Arnold et al. 2001; Kim et al. 1998; Rohmer et al. 2003). Thus, it is possible that selection pressure for the inactivation of an effector may result from the acquisition of new gene functions in both the host and the bacterium and that loss of effector function may be an important factor in the evolution of *P. syringae* virulence. Loss of gene function has been a contributing factor to the evolution of virulence of many pathogens (Ochman and Moran 2001), and the existence of disrupted effector genes has been observed before. For example, analyses of the *hopX1* (*avrPphE*) alleles in *P. syringae* pv. *phaseolicola* and of the exchangeable effector loci in multiple *P. syringae* pathovars have provided snapshots of the disruption of a few effectors in multiple strains and pathovars (Deng et al. 2003; Stevens et al. 1998).

Here, we document the lack of function for multiple effector genes in a single strain. We have shown that several DC3000 effectors are unlikely to be active for various reasons: i) the *hopAG* and *hopS1* ORFs were disrupted by transposable elements; ii) *hopAII*, *hopAH1*, *hopO1-3'*, *hopT1-2*, *hopAS1'*, *hopAH2-1*, *hopAH2-2*, and PSPTO0907 do not appear to be expressed during infection; and iii) HopT2, HopQ1-2, and HopAC1 are not translocated even when constitutively expressed. To test the possibility that the apparent inactivity of these effectors was beneficial to DC3000, we restored several to function, as confirmed by translocation of appropriate Hop-Cya hybrids into *N. benthamiana* cells. However, none of these newly activated effectors had any observable effect on the ability of DC3000 to grow or produce symptoms in *Arabidopsis* Col-0 or tomato cv. MoneyMaker. It is possible that the C-terminal Cya fusion interfered with the function of one or more of these test Hops. However, we have not observed the presence of Cya to disrupt the functions of several other effectors (Badel et al. 2006; Schechter et al. 2004). It also is possible that an effect would be seen if we tested a wider range of host plants among tomato cultivars and related species or among different members of the Brassicaceae. In addition, these effector genes may simply be relics of previous horizontal acquisition events (possibly involving multiple effectors) and these particular effector genes may have no relevance to DC3000 virulence. Nevertheless, it is important to note that a significant portion of the DC3000 effector inventory may not be active. Interestingly, all of the *hop* genes encoded in the conserved effector locus and on plasmid pDC3000A appear to be active. Thus, the effectors that are likely to be most important in DC3000 include those that are most conserved in all *P. syringae* strains (Alfano et al. 2000), as well as those that are likely to be highly mobile.

Analysis of the complete inventory of type III effector genes in *P. syringae* pv. tomato DC3000.

For purposes of guiding future research, effectors can be divided into three categories. First are those that are demonstrably active based on a mutant phenotype or likely active because of native promoter translocation tests or because of microarray and Cya-translocation data indicating strong HrpL-dependent expression and translocation (Chang et al. 2005; Ferreira et al. 2006; Schechter et al. 2004). These are high-priority targets for

research on effector function in pathogenesis. Second are effector genes that are members of validated effectors families but are unlikely to be active in DC3000 because of lack of expression, insertion or frameshift mutations, or inability to be translocated. These inactive genes are of interest for evolutionary studies. The third category includes Hop proteins that are produced more weakly or which may function primarily in the apoplast. These invite further study of deployment and function to establish their status. We generally have little insight into the significance of the greatly varying levels of expression or translocation of various Hops. Nevertheless, our data suggest that the inventory of *hop* genes (including those encoding both true effectors and helpers, but excluding HrpA, HrpK, and other extracellular components of the T3SS machinery) is nearly complete at 53. As summarized in Table 3, 33 of these are likely to be active effectors or apoplast-targeted Hops, 12 are likely to be inactive, and 8 may or may not be produced at functional levels. Almost all of these *hop* genes are associated in the DC3000 genome with Hrp promoters (albeit separated by mobile genetic elements in some cases or associated with Hrp promoters only in other strains in other cases), which facilitates their identification by bioinformatic means. The challenge now is to learn why DC3000 has such a complex inventory of effector genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The *P. syringae* strains used in this study are listed in Table 4 and were grown in King's B medium (KB) at 30°C (King et al. 1954) or *hrp*-derepressing fructose minimal media (Huyh et al. 1989) at 22°C. *Escherichia coli* strains used for cloning and propagating plasmids were grown in Luria-Bertani or Terrific Broth at 37°C (Miller 1992). Antibiotics were used at the following concentrations: chloramphenicol, 20 µg/ml; cycloheximide, 2 µg/ml; kanamycin, 50 µg/ml; rifampin, 50 µg/ml; and spectinomycin, 50 µg/ml.

DNA manipulations.

Plasmid DNA was isolated and manipulated according to standard protocols (Sambrook et al. 1989). PCR was performed with either ExTaq (Takara; Otsu, Shiga, Japan) or Vent (New England Biolabs, Ipswich, MA, U.S.A.). DNA sequencing was performed by the Cornell BioResource Center with a 3700 DNA analyzer (Applied Biosystems; Foster City, CA, U.S.A.).

Construction of plasmids and bacterial mutants.

Plasmids expressing Cya fusion proteins were constructed by Gateway cloning technology (Invitrogen). Candidate effector genes were amplified from DC3000 chromosomal DNA by PCR using the relevant primers listed in Table 5, and the products were cloned into the Gateway entry vector pENTR/SD/D-TOPO (Invitrogen). The candidate effector genes contained in these plasmids were sequenced to confirm that no mutations were present. Recombination (or LR) reactions between each entry vector and the destination vector pCPP3234 (Schechter et al. 2004) then were performed to create the pVLT35-based expression plasmids described in Table 4. These plasmids were maintained in *E. coli* TOP10 (Invitrogen). The transposable elements in *hopAG* and *hopSI* were removed by crossover PCR as described previously (Link et al. 1997) before these genes were cloned into pENTR/SD/D-TOPO.

P. syringae strains containing chromosomal IPET fusions to effector gene promoters were constructed using previously described methods (Boch et al. 2002). The relevant promoter regions were amplified by PCR using the primers listed in Table 5 and ligated to pIPET digested with *KpnI* and *XhoI*. Because pIPET has an R6K origin of replication that depends on the π protein, these plasmids were maintained in DH5 α (*λpir*). Next, the pIPET derivatives were conjugated into *P. syringae* TLR1, and strains containing integrated pIPET plasmids were selected by plating on KB-rifampin-spectinomycin-kanamycin medium. Correct integration of the plas-

Table 4. *Pseudomonas syringae* strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype ^a	Source
<i>P. syringae</i> pv. <i>tomato</i> strains		
DC3000	Wild type, Rif ^r	Cuppels 1986
CUCPB5114	DC3000 Δ <i>hrpK-hrpR</i> ::ΩCm, Rif ^r Cm ^r	Fouts et al. 2003
UNL134-1	DC3000 Δ <i>hrpL</i> ::ΩSp, Rif ^r Sp ^r	Ferreira et al. 2006
TLR1	DC3000 Δ <i>hrcC</i> ::ΩSp, Rif ^r Sp ^r	Boch et al. 2002
JB200	TLR1 <i>avrPto</i> ^{for} ::pIPET, Rif ^r Sp ^r Kan ^r	Boch et al. 2002
JB201	TLR1 <i>avrPto</i> ^{rev} ::pIPET, Rif ^r Sp ^r Kan ^r	Boch et al. 2002
CUCPB5430	TLR1 <i>hopAS1</i> '::pIPET, Rif ^r Sp ^r Kan ^r	This work
CUCPB5431	TLR1 <i>hopR1</i> ::pIPET, Rif ^r Sp ^r Kan ^r	This work
CUCPB5432	TLR1 <i>hopAH2-1</i> ::pIPET, Rif ^r Sp ^r Kan ^r	This work
CUCPB5433	TLR1 <i>hopAH2-2</i> ::pIPET, Rif ^r Sp ^r Kan ^r	This work
Plasmids		
pCPP3234	pVLT35 containing Gateway reading frame B cassette and codons 2 to 406 of <i>cya</i> , Sp ^r /Str ^r Cm ^r	Schechter et al. 2004
pCPP3450	pCPP3234 expressing HopY1-Cya	This work
pCPP3458	pCPP3234 expressing HopR1(201)-Cya	This work
pCPP3470	pCPP3234 expressing HopAH2-1-Cya	This work
pCPP3471	pCPP3234 expressing HopAH2-2-Cya	This work
pCPP3476	pCPP3234 expressing HopB2(400)-Cya	This work
pCPP3491	pCPP3234 expressing HopAG'-Cya	This work
pCPP3492	pCPP3234 expressing HopS1'-Cya	This work
pCPP5147	pCPP3234 expressing HopAS1'-Cya	This work
pCPP5119	pCPP3234 expressing HopAH1-Cya	This work
pCPP5120	pCPP3234 expressing PSPT00907-Cya	This work
pCPP5121	pCPP3234 expressing HopQ2-Cya	This work
pCPP5129	pCPP3234 expressing HopA11-Cya	This work
pCPP5130	pCPP3234 expressing HopT2-Cya	This work
pCPP5131	pCPP3234 expressing HopO1-3'-Cya	This work
pCPP5133	pCPP3234 expressing HopT1-2-Cya	This work
pIPET	Suicide plasmid carrying a promoterless copy of <i>hrcC</i> , Kan ^r	Boch et al. 2002

^a Cya = *Bordetella pertussis* adenylate cyclase. Rif^r, Cm^r, Sp^r, Kan^r, and Str^r = rifampin-, chloramphenicol-, spectinomycin-, kanamycin-, and streptomycin-resistant, respectively.

mids was confirmed by two PCR reactions using primers that flanked each end of the insertion site.

Adenylate cyclase assays.

cAMP levels in infected *N. benthamiana* leaf tissue were determined as described previously (Schechter et al. 2004). Briefly, *P. syringae* strains were suspended to an optical density at 600 nm of 0.3 (1×10^8 CFU/ml) in a solution containing 5 mM morpholinoethanesulfonic acid (pH 6.0) and 100 μ M IPTG. Bacteria were infiltrated into plant tissue with a blunt syringe, and samples were collected 7 h post inoculation with a 0.8-cm-diameter cork borer. Leaf disks then were frozen in nitrogen, ground to a powder, and suspended in 300 μ l of 0.1 M HCl. cAMP was quantified using the Correlate-EIA cAMP immunoassay kit (Assay Designs, Ann Arbor, MI, U.S.A.) and protein levels were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA, U.S.A.) according to the manufacturer's directions. As noted previously (Schechter et al. 2004), some batches of plants can yield higher or lower levels of cAMP in Cya translocation assays for all test strains. Therefore, it is important to include reference strains in each experiment for quantitative comparisons.

Real-time PCR.

Real-time PCR was performed in an ABI 7000 Sequence Detection System (Applied Biosystems) and is described in detail elsewhere (Vencato et al. 2006). Briefly, PCR reactions contained iTaQ Sybr Green Supermix with Rox (Bio-Rad), *P. syringae* cDNA, and 0.3 μ M of the relevant primers listed in Table 5. The cDNA in the PCR reactions was reverse tran-

scribed from total RNA using the cDNA synthesis kit (Bio-Rad). Total RNA was extracted from *P. syringae* using the SV Total RNA Isolation System (Promega Corp., Madison, WI, U.S.A.) and the RNeasy Minikit (Qiagen, Hilden, Germany) (Vencato et al. 2006). The efficacy of all primer pairs was confirmed using DC3000 DNA templates. The threshold cycle (Ct) value of each gene tested was normalized to the Ct values of two housekeeping genes, *gyrA* (PSPTO1745) and *gap1* (PSPTO1287), separately, and these values then were averaged to obtain relative expression data for each gene in wild-type DC3000 and *hrpL* mutant strains.

Virulence and HR assays.

P. syringae growth in planta was determined as previously described (López-Solanilla et al. 2004). Briefly, tomato plants (*Solanum lycopersicum* cv. Money Maker) were grown under greenhouse conditions for 3 to 4 weeks and dipped for 30 s in a 10 mM MgCl₂, 0.02% Silwet solution containing the test strains at 5×10^6 CFU ml⁻¹. Infected plants were maintained in a growth chamber at 25°C with illumination (16 h of light and 8 h of dark) during the course of the experiment. At various time points, 10 leaf samples were collected with a 0.7-cm boring tool and weighed. The leaf tissue then was homogenized in 0.5 ml of 10 mM MgCl₂ and bacterial counts were determined by plating a series of dilutions on KB-rifampin-cycloheximide medium.

For HR assays, *N. tabacum* cv. Xanthi plants were grown under greenhouse conditions for 4 to 6 weeks. Bacteria were infiltrated into leaf tissue using a blunt syringe, and plants were maintained in the laboratory with illumination at room temperature (24°C).

Table 5. Primers used in this study

Gene	5' (upstream) primer	3' (downstream) primer
For construction of <i>cya</i> fusions ^z		
<i>hopY1</i> (PSPTO0061)	5'-CACCATGAACATTACGCCGCTCAGC-3'	5'-CTGGTAGTTGATGCCCGTGG-3'
<i>hopAS1'</i> (PSPTO0474)	5'-CACCATGACCTTAAGAATCAATAC-3'	5'-AGAAAACCTCGCTTTCTGTT-3'
<i>hopR1</i> (PSPTO0883)	5'-CACCATGGTCAAGGTTACCTCTTCC-3'	5'-GGGATTGATGCCTGGCAGCG-3'
<i>hopAH1</i> (PSPT00905)	5'-CACCATGAGTATGAATACCTCTGTAAGC-3'	5'-GGAGAATTGATCCCCTTTCG-3'
<i>hopAII</i> (PSPTO0906)	5'-CACCATGCTCGCTTTGAAGCTGAA-3'	5'-GCGAGTCCAGGGCGGTGGCA-3'
PSPTO0907	5'-CACCATGCCACATCCAGTCTCTCA-3'	5'-GCGTTCGCCATAAGCAAGCC-3'
<i>hopAH2-1</i> (PSPTO3292)	5'-CACCATGAATATCAATTCAATCCGGTC-3'	5'-GTATTGATCTGACTTTGAAAATCCGAC-3'
<i>hopAH2-2</i> (PSPTO3293)	5'-CACCATGAACATCCACACCGCC-3'	5'-TGAGTACTGATCTGACTTTGAAAATCCG-3'
<i>hopO1-3'</i> (PSPTO4592)	5'-CACCATGAATATTAACCCCTTC-3'	5'-GTGTTGATACCGAAGATGAT-3'
<i>hopT1-2</i> (PSPTO4593)	5'-CACCATGATCAAAACAGTCAGCCGA-3'	5'-GCCTACCCGATCAGCGGCTG-3'
<i>hopAC</i> (PSPTO4996)	5'-CACCATGTGGGGTTCGTGGATC-3'	5'-CGAGCTGAACGGTCCGACCT-3'
<i>hopT2</i> (PSPTO4590)	5'-CACCCTGTGTTTCCCGCC-3'	5'-CCATGACGCTTTATTGGCTG-3'
<i>hopQ1-2</i> (PSPTO4732)	5'-CACCATGAGCAGCCAGCTTTGGA-3'	5'-ATCCGGGACTGCCGTCGACT-3'
<i>hopAG'</i> (PSPTO0901)	5'-CACCATGAACCTATAACACACAGCTTTAG-3'	5'-CGGTATAAACACCTTTAACTACCCGCAAGCC-ATGG-3'
<i>'hopAG</i> (PSPTO0904)	5'-CCATGGCTTGCGGGTAGTTAAAGGTGTTTAT-ACCG-3'	5'-GAAGGGGTAAAGCCTGAAAACC-3'
<i>hopSI'</i> (PSPTO4597)	5'-CACCATGAATGCGTTCGCAACCG-3'	5'-CGTCGTCATCAAGGTGACCAAGCTCTGGA-TTTTATC-3'
<i>'hopSI</i> (PSPTO4595)	5'-GATAAAAATCCAGAGCTTGGTCACCTTGA-TGACGACG-3'	5'-TATGCCACCTTCCCGAGTCTG-3'
For RT-PCR		
<i>hopAS1'</i> (PSPTO0474)	5'-TCCGAACGTCAGACCAATTTTC-3'	5'-TCGATACCTGCTCACCTTCCTC-3'
<i>hopR1</i> (PSPTO0883)	5'-GCAAATACAAGTGCAGCTGGG-3'	5'-TCTGGTTCAGGTTTACGCGG-3'
<i>hopAH1</i> (PSPTO0905)	5'-TTGATCAAGTCTGCGCGTGA-3'	5'-CGTATTTGACCAGTCCGCTTTC-3'
PSPTO0907	5'-CCACATCGAAACACAGTTGGC-3'	5'-CCTGTCGACCTTTCAACAGA-3'
<i>hopAH2-1</i> (PSPTO3292)	5'-GGTCAAAAAATTCGGCGGTC-3'	5'-TCAGCGTACTTTCCATGTCAGC-3'
<i>hopAH2-2</i> (PSPTO3293)	5'-CCACCAAGATGATTCAGGACCT-3'	5'-AGCTTGCCATCTGCTTGACGT-3'
<i>hopO1-3'</i> (PSPTO4592)	5'-CTGTTTTTTCGCCGTGCCTT-3'	5'-CCCCTGAATGTTGCTGGAGTAA-3'
<i>hopT1-2</i> (PSPTO4593)	5'-CGGATGGAAATCTGTACGCA-3'	5'-CCGAGGTTTGAAGCAGACTCAA-3'
For cloning promoters into pIPET		
<i>hopAS1'</i> (PSPTO0474)	5'-CCGGTACCATCTTTCTTGCAACCTTCATCAC-3'	5'-GGCTCGAGCGGGCTCCATCGGCT-3'
<i>hopR1</i> (PSPTO0883)	5'-GAGGTACCGTGGAGGTTGATAGGCA-3'	5'-GGCTCGAGATCGTTATTATTAACCTCTG-3'
<i>hopAH2-1</i> (PSPTO3292)	5'-GAGGTACCGTGACCCGATTGGCAGTG-3'	5'-GCCTCGAGAGCGTCTCTTTCTGAGC-3'
<i>hopAH2-2</i> (PSPTO3293)	5'-GCGGTACCAGGACACCAAGGAGATG-3'	5'-GGCTCGAGAATGTCTCTGTGGGCTCTG-3'

^z Cya = *Bordetella pertussis* adenylate cyclase; RT-PCR = real-time polymerase chain reaction; pIPET = in planta expression technology.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Pseudomonas–Plant Interaction website: pseudomonas-syringae.org