Genome-Assisted Development of a Diagnostic Protocol for Distinguishing High Virulence *Pseudomonas syringae* pv. *tomato* Strains

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Abstract

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A severe outbreak of bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, occurred in central New York in 2009. Isolate 09150, collected from this outbreak and subsequently named NYS-T1, was found to be highly virulent on tomato. To better understand the relationship of 09150 to other *P. syringae* strains and develop a diagnostic assay for aggressive strains of this pathogen, the 09150 genome was sequenced. Genome comparison revealed it to be highly similar to a previously sequenced isolate, T1. Genetic factors linked to host interaction including type III effectors, toxin biosynthetic genes, and elicitors of host innate immunity were identified. Type III effector repertoires were compared with other strains in the high virulence T1-like subgroup and

lower virulence DC3000/*P. syringae* pv. *maculicola* subgroup within *P. syringae* phylogenetic Group I. Primers for conventional PCR were developed using sequences for *avrA*, *hopW*, conserved in the former subgroup and *hopN*, present in the latter. These were tested on isolates in the two subgroups, other pseudomonads, and other bacterial pathogens of tomato. Primers developed for *avaA* and *hopW* were diagnostic for more virulent strains of *P. syringae* pv. *tomato* while primers for *hopN* were diagnostic for *P. syringae* pv. *tomato* DC3000 and related *P. syringae* pv. *maculicola* strains. Primers designed against *hopR* distinguished both of these *P. syringae* subgroups from other *P. syringae* strains.

Pseudomonas syringae pv. *tomato* is the causal agent of bacterial speck disease of tomato (*Solanum lycopersicum* L.) and affects tomato production worldwide (28). Bacterial speck can be severe and lead to substantial economic losses (29,48). The pathogen is disseminated through infested seed and can survive in infected debris and epiphytically on weeds (35). For tomatoes grown commercially in New York, new strains typically arrive on infected seed or transplants. Virulence can vary widely among *P. syringae* pv. *tomato* strains and several other bacterial pathogens of tomato cause symptoms similar to those of *P. syringae* pv. *tomato*, complicating management of bacterial speck (30). Diagnostics specific to the more virulent *P. syringae* pv. *tomato* could enhance disease management practices.

During the late summer of 2009, a highly virulent strain of *P. syringae* pv. *tomato* (09150) was isolated from an Ontario County, NY tomato field. The severity of this isolate, subsequently named NYS-T1, resulted in total loss of yield from a 5-acre field, prompting interest in development of diagnostic tools that could be used to distinguish highly virulent *P. syringae* pv. *tomato* strains from milder *P. syringae* strains as well as from other bacterial pathogens causing speck-like symptoms. This latter group includes *Xanthomonas* spp. (*Xanthomonas vesicatoria*, *X. euvesicatoria*, *X. gardneri*, and *X. perforans*) causing bacterial spot of tomato and *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial wilt and canker of tomato (19,22,24). *P. viridiflava*, the causal agent of bacterial leaf blight and stem pith necrosis of tomato, can also cause speck-like symptoms (23,34) and was found in 2010 on the same farm from which *P. syringae* pv. *tomato* 09150 had been isolated.

DNA-based detection of *P. syringae* pv. *tomato* strains from infected tomato has typically been conducted using sequences of

hrpZ and other genes in the *hrp/hrc* gene cluster that do not distinguish among strains having different virulence levels (20,21,49). Genes involved in biosynthesis of the phytotoxin coronatine have also been used for DNA-based detection (16,36), although coronatine biosynthesis is not strongly correlated with either strain phylogeny or the degree of virulence (5). A detection protocol capable of distinguishing more virulent *P. syringae* pv. *tomato* strains from both less virulent strains and other bacterial pathogens associated with tomato would aid growers in making pest management decisions when faced with an early stage infection in the field.

Several *P. syringae* strains, most notably *P. syringae* pv. *tomato* DC3000, have been developed as model systems for genomics-enabled characterization of bacterium-host interactions. Consequently, there is a wealth of sequence data available for use in development of DNA-based diagnostic tools. Information on complete and draft genomes of *P. syringae* isolates and related *Pseudomonas* species can be found at the *Pseudomonas*-Plant Interaction Resource (www.pseudomonas-syringae.org) and at www.pseudomonas.com with an extensive catalog of sequences for conducting MLST analysis available at the Plant Associated and Environmental Microbes Database (www.PAMDB.org). *P. syringae* strains isolated from agricultural settings fall into three major phylogenetic groups with pathovars *tomato*, *maculicola*, and *actinidae* assigned to “Group I” (4,40). Included in Group II are the *P. syringae* pathovars *syringae* and *pisi* and Group III pathovar examples are *glycinea*, *phaseolicola*, and *aesculi*. Multilocus sequence typing of Group I strains further reveals that more virulent isolates of *P. syringae* pv. *tomato*, exemplified by strain T1, cluster together (10,47). In contrast, strain DC3000 belongs to a different subgroup, which includes strains more closely related to *P. syringae* pv. *maculicola*. The two subgroups are referred to here as “T1-like” and “DC3000/*P. syringae* pv. *maculicola*.” Strains in the latter subgroup generally have a broader host range extending to *Arabidopsis thaliana* (L.), cauliflower (*Brassica oleracea* L. var. *botrytis*), and other brassicas, while those in the T1-like subgroup cause disease on tomato but not cauliflower or *Arabidopsis* (15,46,47). Analysis of strains collected over a 70-year time period indicates that in Europe and North America, the T1-like subgroup

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has been isolated with increasing frequency relative to DC3000/*P. syringae* pv. *maculicola* strains (10).

To better understand how the field isolate 09150 corresponds to the two subgroups and to identify sequence signatures that reliably distinguish high and low virulence strains, isolate 09150 was sequenced and the repertoire of predicted virulence determinants compared with those of related *P. syringae* strains. Virulence factors receiving particular attention included type III effector (T3E) repertoires, phytotoxins, and gene products linked to induction of innate immunity in the host. Here we report these virulence factors and on a PCR-based subspecific diagnostic assay developed from genomic comparison of *P. syringae* pv. *tomato* strains with varying levels of virulence.

Materials and Methods

Bacterial cultures and DNA extraction. Bacterial isolates used in this study were obtained from frozen cultures stored at -80°C from the culture collections in the laboratories of C. Smart and A. Collmer at Cornell University (Table 1). Isolates of *Pseudomonas* spp., *X. perforans*, and *C. michiganensis* were maintained on King's B agar, yeast dextrose carbonate agar, and D2ANX agar, respectively, at 28°C (13,27,46). For DNA extraction, overnight cultures were grown in Luria-Bertani (LB) broth at 28°C on a rotary shaker at 200 rpm (6). DNA extraction was performed according to the protocol by Chen and Kuo (11). For DNA extraction of *P. syringae* pv. *tomato* from infected and healthy tomato tissue, the protocol from Keb-Llanes et al. (25) was used with modifications (polyvinylpyrrolidone and β -mercaptoethanol were omitted from extraction buffer A).

Genomic sequencing of *P. syringae* pv. *tomato* 09150. Genomic DNA from *P. syringae* pv. *tomato* 09150 was extracted according to the protocol from Chen and Kuo (11) with modification (an extraction with an equal volume phenol/chloroform/isoamyl alcohol [25:24:1] was performed with the supernatant from cell lysis after separation from cellular debris) and was sequenced using Illumina HiSeq (Illumina, Inc., San Diego, CA) at the Cornell University's Genomics Core Facility in Ithaca, NY. Initial coverage of 2,434x

was reduced to 2,013x after quality trimming and error correction with Quake (version 0.2.2) (26). De novo assembly was conducted using Velvet (version 1.1.04) (50) and mapping assembly against the *P. syringae* pv. *tomato* T1 reference genome with MIRA (version 3.4.0 prod_linux-gnu_x86_64_static) (12). The assemblies were manually analyzed using MAUVE (17). Overlapping contigs were merged using Minimus2 from the AMOS package (version 3.0.0., www.sourceforge.net). A final round of scaffolding was performed using SSPACE (version 1.1) with default parameters (8). Genome annotation of the resulting 188 contigs was conducted with RAST (3) and HrpL binding sites were predicted with Pred_cutoff (38). To determine the phylogenetic placement of strain 09150, sequences corresponding to the 9-gene MLST schema described by Almeida et al. (1) were identified from the 09150 genome assembly. Sequences used for the MLST analyses were obtained from the Plant Associated and Environmental Database (www.PAMDB.org) for *P. syringae* pv. *tomato* isolates Max13, kuzzen 40, T1, NCPPB 1108, JL1065, Pan 126, DC3000, ICMP3443; *P. syringae* pv. *maculicola* isolates M3 and M6; and *P. syringae* pv. *syringae* B728a. Sequences for *P. viridiflava* UASWS0039, *P. fluorescens* NCIMB 11764, *P. syringae* pv. *glycinea* B076, and *P. syringae* *aesculi* 2250 were obtained from the National Center for Biotechnology (www.ncbi.nlm.nih.gov). A phylogenetic tree was produced using the neighbor-joining method in MEGA6 (39,41). The evolutionary distances were computed using the Maximum Composite Likelihood method (42). The GenBank accession number for the NYS-T1 sequence data are JRRRA000000000. Additional genome resources for NYS-T1 can be found on the *Pseudomonas*-Plant Interaction web site at http://pseudomonas-syringae.org/Pto-NYS-T1_Genome_Resources.html.

Primer design. Sequences for T3E genes, *avrA*, *hopW1*, *hopN1*, and *hopR1*, were aligned using MEGA6 (Table 3). Primer sequences were designed for each gene using a visual assessment of aligned sequences combined with Integrated DNA technologies (IDT, Coralville, IA) OligoAnalyzer tool (<https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>).

Table 1. Bacterial isolates used in this study

Species	Isolate	Host of isolation	Origin	Year	Source	Reference
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	0578 ^z	<i>Solanum lycopersicum</i>	NY, USA	2005	C. Smart	This study
	0761 ^z	<i>S. lycopersicum</i>	NY, USA	2007	C. Smart	This study
	09084 ^z	<i>S. lycopersicum</i>	NY, USA	2009	C. Smart	This study
	09150 ^z	<i>S. lycopersicum</i>	NY, USA	2009	C. Smart	This study
	12042 ^z	<i>S. lycopersicum</i>	NY, USA	2012	C. Smart	This study
	13093	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study
	13110	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study
	K40 ^z	<i>S. lycopersicum</i>	USA	2005	A. Collmer	10
	A9 ^z	<i>S. lycopersicum</i>	USA	1996	C. Smart	21
	JL1065 ^z	<i>S. lycopersicum</i>	CA, USA		A. Collmer	44
	NCPBP 1108	<i>S. lycopersicum</i>	Jersey, UK	1961	A. Collmer	
	Max13	<i>S. lycopersicum</i>	France		A. Collmer	49
	T1	<i>S. lycopersicum</i>	Canada		A. Collmer	37
	DC3000 ^z	<i>S. lycopersicum</i>	Geurnsey, UK	1961	A. Collmer	14
<i>P. syringae</i> pv. <i>antirrhini</i>	Pat126 ^z	<i>Antirrhinum majus</i>		1965	A. Collmer	47
<i>P. syringae</i> pv. <i>maculicola</i>	M3 ^z	<i>Brassica oleracea</i> var. <i>botrytis</i>	USA	1937	A. Collmer	18
	M6 ^z	<i>B. oleracea</i> var. <i>botrytis</i>	UK	1965	A. Collmer	18
<i>P. syringae</i> pv. <i>syringae</i>	B728 ^z	<i>Phaseolus vulgaris</i>	WI, USA		C. Smart	33
<i>P. viridiflava</i>	10078 ^z	<i>S. lycopersicum</i>	NY, USA	2010	C. Smart	This study
<i>P. fulva</i>	0430 ^z	<i>S. lycopersicum</i>	NY, USA	2004	C. Smart	This study
<i>P. fluorescens</i>	09110 ^z	<i>S. lycopersicum</i>	NY, USA	2009	C. Smart	This study
<i>P. putida</i>	09112 ^z	<i>S. lycopersicum</i>	NY, USA	2009	C. Smart	This study
<i>Xanthomonas perforans</i>	13091	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	13048	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study
	13085	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study
	13117	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study
	13129	<i>S. lycopersicum</i>	NY, USA	2013	C. Smart	This study

^z Isolates used in virulence assay. All isolates were used in primer development.

Primer testing. Each primer combination (Table 4) was tested using the Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). A temperature gradient of 56 to 65°C was used to determine the optimum annealing temperature for each primer pair. Twenty-five microliter reactions contained 1× EmeraldAmp PCR MasterMix (Clontech, Mountain View, CA), 10 pmol forward primer, 10 pmol reverse primer, and 10 ng template DNA. Multiplex PCR was performed with primers for *avrA* (215f and 1321r) and *hopR1* (427f and 1129r) with the same reaction mixture as above except that 7.5 pmol of each primer was used. PCR conditions were as follows: initial denaturation of 94°C for 4 min, followed by 35 cycles at 94°C for 45 s, 56 to 65°C for 30 s, 72°C for 45 s, and a final extension step of 72°C for 5 min. PCR products were electrophoresed for 60 min at 100 V on a 1%

Table 2. Predicted virulence factors for *P. syringae* pv. *tomato* DC3000, T1, and NYS-T1 from genomic data. X's indicate the presence of full length reading frames

	DC3000	T1	NYS-T1
Type III effectors			
AvrE1	X	X	X
HopC1	X	X	X
HopH1	X	X	X
HopI1	X	X	X
HopR1	X	X	X
HopO1-2	X	X	X
HopS2	X	X	X
HopT1-2	X	X	X
HopY1	X	X	X
HopAA1-1	X	X	X
HopF2	X	X	X
HopD1	X	X	X
HopQ1	X	X	X
HopO1-1	X	X	X
HopT1-1	X	X	X
HopAF1	X	X	X
HopW1		X	X
AvrA		X	X
HopAS1		X	X
HopS1		X	X
HopAE1		X	X
HopAG1		X	X
HopAH1		X	X
HopAI1		X	X
AvrRpt2		X	X
AvrD1		X	X
HopAB2(AvrPtoB)	X		
HopA1	X		
HopB1	X		
HopM1	X		
HopN1	X		
HopE1	X		
HopG1	X		
HopV1	X		
HopAA1-2	X		
HopAD1	X		
HopAM1 (2 copies)	X		
HopAO1	X		
HopAQ1	X		
AvrPto1	X		
HopAO-like candidate			X
Hrp regulon			
PSPTO_0834-0836	X		
PSPTO_4733	X		
Toxins			
coronatine	X		X
PAMP elicitation			
flgII-28 epitope of FliC	X	X	
Other			
type II restriction			X

agarose gel stained with ethidium bromide. Primers were tested with DNA from all isolates listed in Table 1, infected tomato leaf tissues (*P. syringae* pv. *tomato* 09150 and DC3000), infected tomato fruit (*P. syringae* pv. *tomato* 13093 and 13110), and healthy tomato leaves. Infected and healthy tomato leaf tissues were collected from cultivar Mountain Fresh Plus. Infected tomato fruit tissues were obtained from field samples collected from unknown cultivars.

Virulence assay. Five-week-old tomato plants ('Mountain Fresh Plus') were inoculated with pseudomonads (Table 1) from overnight bacterial cultures grown in LB broth at 28°C on rotary shaker (200 rpm). Inoculum was prepared by adjusting bacterial concentration to an OD600 of 0.05 (approximately 10⁷ CFU/ml) in 50 ml of 1× phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) containing 0.25% Tween. Plants were dip-inoculated and kept in a greenhouse at 24°C (daytime) and 20°C (nighttime) with 14 h light per day. Two plants per isolate were inoculated one repetition and four plants per isolate were inoculated for a second repetition. Plants were rated for disease by counting the number of specks per leaflet on the first two true leaves, starting 6 days post inoculation when specks were first visible and continuing every 2 days for a total of four ratings. Analysis of variance and Tukey's honestly significant differences were calculated from data from the fourth rating (12 days postinoculation) using JMP Pro 10 (SAS Institute, Cary, NC) to determine significant differences ($\alpha = 0.05$) between mean specks per leaflet for each isolate. No significant differences were found between repetitions and data from both repetitions were combined for analysis using a total of six plants per isolate.

Results

Genome annotation reveals predicted virulence determinants encoded by *P. syringae* pv. *tomato* NYS-T1. The overall genome size of 6.26 Mb for *P. syringae* pv. *tomato* NYS-T1 falls within the size range observed for previously sequenced *P. syringae* strains. Multilocus sequence typing of NYS-T1 with the schema described by Yan et al. (47) phylogenetically places it with Group I strains, clustering most closely with four strains in the T1-like subgroup (Fig. 1). To determine whether NYS-T1 encodes a novel combination of virulence associated genes and to facilitate broader comparison of virulence gene repertoires among *P. syringae* strains pathogenic on tomato, candidate virulence genes were identified through sequence comparison and de novo identification of regulatory motifs. Of particular interest were genes linked to phytotoxin biosynthesis, genes encoding Type III effectors (T3Es), and variations in the predicted protein sequences of the conserved pathogen associated molecular patterns (PAMPS) that

Table 3. Sequences from *P. syringae* pv. *tomato* isolates used for primer design

Gene	Strain	Accession
<i>avrA</i>	NCPPB1108	ADGA01000238.1
	T1	ABSM01000053.1
	K40	ADFY01000462.1
	Max13	ADFZ01000171.1
	09150	this study
<i>hopW1</i>	NCPPB1108	ADGA01000243.1
	T1	ABSM01000048.1
	K40	ADFY01000403.1
	Max13	ADFZ01000107.1
	09150	this study
<i>hopN1</i>	DC3000	AE016853.1
<i>hopR1</i>	NCPPB1108	ADGA01000036.1
	T1	ABSM01000021.2
	K40	ADFY01000071.1
	Max13	ADFZ01000207.1
	09150	this study
	DC30000	AE016853.1

trigger host immunity. The T3E repertoires of T1 and NYS-T1 appear to be identical (with a possible exception discussed below), consistent with previous observations of high overall genome conservation among T1-like strains (10). Predicted HrpL regulated genes that are not T3Es are similarly conserved between T1 and NYS-T1, with both strains lacking genes corresponding to PSPTO_4733 and PSPTO_0834-0836 in the non-T3E HrpL regulon of DC3000.

Four differences between strains T1 and NYS-T1 were identified with likely or potential impact on host interaction. Chief among these is the presence of biosynthetic genes for the phytotoxin coronatine in the NYS-T1 genome. Another feature with an experimentally confirmed role in virulence is the presence of a sequence variation in the flgII-28 epitope of NYS-T1 FliC protein, linked to reduced induction of PAMP-triggered immunity in tomato (10).

Table 4. Primers designed for *P. syringae* pv. *tomato* type III effectors

Gene	Primer name and position ^z	Sequence	Annealing temp °C
<i>avrA</i>	547f	5'-CGATCTCTGTCTGAACAATGC-3'	65
	215f	5'-CGCATGTTTCAGCATTGTCAT-3'	65
	1321r	5'-GAAGACCTTGGTTCTTTCGG-3'	65
	1219r	5'-TTGCCTGGTCGATTGTCAAC-3'	65
<i>hopW1</i>	173f	5'-AGGACTTCACAAGCCTTCTG-3'	65
	313f	5'-GAACAGCAGACACTCAAAGG-3'	56
	1099r	5'-CCTGTGTCCAATTTGTCCTC-3'	65
	1327r	5'-CGTCTACGACCTTACCATCG-3'	56
<i>hopN1</i>	91f	5'-AATGGAAGCGAGTGTCTGC-3'	58
	142f	5'-TCTTCACACAGATCGAAAGGC-3'	58
	771r	5'-CGATAGAGACCATCAGATCCG-3'	58
	816r	5'-GATTCTGGTCTTGATGTATTGCG-3'	58
<i>hopR1</i>	427f	5'-GAGATGGAACATGGCATCAG-3'	65
	705f	5'-GATGGTGGAGTCTATCTGC-3'	65
	1129r	5'-AGGTGAACAGTGTCTGCTC-3'	65
	1299r	5'-CATGACCATCAAGCTGAACG-3'	65

^z Position corresponds to the 5' end of the primer in reference to the adenine of the start codon of the corresponding gene, f designates forward primers and r designates reverse primers.

Fig. 1. Evolutionary history of *Pseudomonas* spp. using the neighbor-joining method. Concatenated sequences and group designations are described by Yan et al. 2008 (47). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Additional NYS-T1 features of potential interest include a candidate novel effector having 34% identity over a 290 amino acid region with the T3E HopAO1, and a type II restriction system that is similarly absent from both T1 and DC3000. Comparison of predicted NYS-T1 virulence factors with those in T1 and DC3000 is shown in Table 2.

Identification of T3E gene profiles conserved within the T1-Like and DC3000/*P. syringae* pv. *maculicola* subgroups. To determine the suitability of T3E genes as target loci in the development of DNA-based diagnostic methods, conservation of these genes among sequenced strains in the T1-like subgroup was evaluated. T3E repertoires were predicted from the draft genomes of three T1-like strains K40, NCPPB1106, and Max13 collected at different times and in different geographical regions (10). Comparison with T1 and NYS-T1 revealed a high degree of T3E gene conservation, with 19 effectors conserved in all five strains. Among those present in the five T1-like strains but either entirely absent or truncated in the DC3000 genome are *hopW1*, *avrA*, and *hopAE1*. The genes

hopW1 and *avrA* were selected for primer development, as *hopAE1* is present in *Pma* strains M3 and M6 in the DC3000/*P. syringae* pv. *maculicola* subgroup (B. Vinatzer, unpublished data). *P. syringae* pv. *tomato* DC3000 has nine effector genes that lack homologs among the sequenced T1-like strains. Of these, *hopN1* was selected as a candidate diagnostic locus for the *Pto* DC3000/*P. syringae* pv. *maculicola* subgroup. The *hopN1* sequences in the Hop Database (www.pseudomonas-syringae.org) were aligned to identify conserved regions.

Primers derived from T3E genes *avrA*, *hopW1*, and *hopN1* effectively discriminate among high and low virulence strains of *P. syringae* pathogenic on tomato. To confirm the efficacy of the *avrA* and *hopW1* genes as diagnostic loci for strains in the T1-like subgroup, four primer combinations derived from conserved regions in these genes were evaluated (Tables 4 and 5). Products of the expected size were amplified from *P. syringae* pv. *tomato* T1, NYS-T1, K40, NCPPB1108, Max13, and JL1065. No products were amplified from either *P. syringae* pv. *tomato* DC3000 or the

Table 5. PCR production amplification from primers developed in this study^x

	Primers	<i>avrA</i>				<i>hopW1</i>				<i>hopN1</i>				<i>hopR1</i>			
		547f 1321r	547f 1219r	215f 1321r	215f 1219r	173f 1099r	173f 1327r	313f 1099r	313f 1327r	91f 771r	91f 816r	142f 771r	142f 816r	427f 1129r	427f 1299r	705f 1129r	705f 1299r
<i>P. syringae</i> pv. <i>tomato</i>	Isolate																
T1 subgroup	T1	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
T1 subgroup	NYS-T1	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
T1 subgroup	Max13	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
T1 subgroup	NCPPB1108	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
T1 subgroup	K40	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
T1 subgroup	A9	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
T1 subgroup	JL1065	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
DC3000/ <i>P. syringae</i> pv. <i>maculicola</i> subgroup	DC3000	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
DC3000/ <i>P. syringae</i> pv. <i>maculicola</i> subgroup	M3	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
DC3000/ <i>P. syringae</i> pv. <i>maculicola</i> subgroup	M6	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
unknown	0578	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
unknown	0761	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
unknown	09084	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
unknown	12042	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
unknown	13093	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
unknown	13110	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
<i>P. syringae</i> pv. <i>antirrhini</i>	Pat126	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
<i>P. syringae</i> pv. <i>syringae</i>	B728a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. viridiflava</i>	10078	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. fulva</i>	0430	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. fluorescens</i>	09110	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. putida</i>	09112	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Xanthomonas</i> <i>perforans</i>	13091	+ ^y	+ ^y	+ ^y	+ ^y	+ ^z	-	-	-	-	-	-	-	-	-	+ ^z	+ ^z
<i>Clavibacter</i> <i>michiganensis</i> subsp. <i>michiganensis</i>	13048	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	13085	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	13117	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	13129	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

^x + Target amplification with corresponding primer pair and isolate; - no amplification with corresponding primer pair and isolate.

^y Weak target amplification at lower annealing temperatures.

^z Weak target amplification at all annealing temperatures.

P. syringae pv. *maculicola* strains M3 or M6 (Table 5, Fig. 2). The primers 215f and 1321r for *avrA* were selected for diagnostics to distinguish T1-like strains from the DC3000/*P. syringae* pv. *maculicola* strains using an annealing temperature of 65°C.

Two primer combinations aligning to conserved regions of *hopN1* were evaluated with members of the T1-like and DC3000/*P. syringae* pv. *maculicola* subgroups. Amplified products of the expected size were apparent for DC3000, *P. syringae* pv. *maculicola* M3 and M6, but not for any of the T1-like strains, confirming the utility of this locus for distinguishing the DC3000/*P. syringae* pv. *maculicola* subgroup (Table 5).

The T3E gene *hopR1* is highly conserved among Group I *P. syringae* strains associated with tomato and was used for development of primers that can generally distinguish these strains from other *P. syringae* groups. As shown in Table 5, all *P. syringae* isolates tested yielded an amplified product with the exception of *P. syringae* pv. *syringae* B728a, which is a member of *P. syringae* Group II.

None of the non-*P. syringae* bacterial pathogens evaluated yielded a *hopR1* amplification product (Table 5, Fig. 2).

Primer sets were used to evaluate the subgroup membership of six *P. syringae* isolates from the Smart lab collection. All showed amplification products using all the *avrA* and *hopR* primer combinations and no products from *hopN* primers, confirming membership of these strains in the T1-like subgroup.

Amplification patterns resulting from a multiplex PCR employing *avrA* primers 215f and 1321r and *hopR1* primers 427f and 1129f are shown in Figure 2. Results show target-sized amplification products for the *avrA* primer set for strains in the T1-like subgroup. Target amplification was seen for *hopR1* primers in both the T1-like and DC3000/*P. syringae* pv. *maculicola* strains. No amplification was observed for the remaining pseudomonads or other bacterial pathogens of tomato. The applicability of the multiplex PCR test for detection of high virulence T1-like in field isolates was demonstrated by use of these primers on infected

Fig. 2. Results of PCR amplification for T3Es. **A**, *avrA* primers 215f and 1321r; **B**, *hopR1* primers 427f and 1129r; and **C**, multiplex with *avrA* primers 215f and 1321r and *hopR1* primers 427f and 1129r. Lanes are as follows: M: DNA ladder (Invitrogen, Low DNA Mass Ladder); (1-11 *P. syringae* pv. *tomato*) 1: 0578; 2: 09084; 3: NYS-T1; 4: 12042; 5: K40; 6: A9; 7: JL1065; 8: T1; 9: Max13; 10: NCPPB1108; 11: DC3000; (12 and 13 *P. syringae* pv. *maculicola*) 12: M3; 13: M6; 14: leaf tissue infected with NYS-T1; 15: leaf tissue infected with DC3000; 16: fruit tissue infected with 13093; 17: fruit tissue infected with 13110; 18: healthy tomato leaf tissue; 19: *P. syringae* pv. *antirrhini* Pat126; 20: *P. syringae* pv. *syringae* B728a; 21: *P. viridiflava* 10078; 22: *P. fulva* 0430; 23: *P. fluorescens* 09110; 24: *P. putida* 09112; 25: *X. perforans* 13091; (26-29 *C. michiganensis* subsp. *michiganensis* strains) 26: 13048; 27: 13085; 28: 13177; 29: 13129; 30: negative water control.

tomato leaf and fruit tissue. An annealing temperature of 65°C is recommended given that weak amplification was observed at lower temperatures in *X. perforans* for some of the primer combinations (Table 5).

Comparative virulence of pseudomonads on tomato. The average number of necrotic specks per tomato leaflet was highest among the T1-like *P. syringae* pv. *tomato* strains NYS-T1, K40, 09084, A9, and T1 (Table 6). *P. syringae* pv. *tomato* DC3000 and *P. viridiflava* 10078 showed average specks per leaflet counts that were not significantly different from the water control at the inoculation level used in these assays (Table 6). The average specks per leaflet for DC3000 was lower than that of those for the T1-like strains, but statistically significant differences were not found between DC3000 and the T1-like strains T1 and A9.

Discussion

Plant pathogen populations are dynamic in structure and distribution, responding to a broad array of variables including the genetic makeup and spatial distribution of the host as well as agronomic practices contributing to spread of infected material. Among Group I *P. syringae* isolates, the more recently evolved T1-like subgroup has become predominant over less virulent, broader host-range strains in the DC3000/*P. syringae* pv. *maculicola* subgroup (10). Continued monitoring of changes in lineage distribution and evolution can benefit from development of reliable and discriminating DNA-based diagnostic assays that, unlike culture-based methods, are capable of detecting differences among phenotypically distinct sublineages. To date, most DNA-based detection of *P. syringae* has relied on primers hybridizing to genes widely conserved among diverse isolates, or against biosynthetic genes for the phytotoxin coronatine, which are unevenly distributed among *P. syringae* pv. *tomato* strains and not consistently correlated with virulence.

The appearance of a highly virulent strain of *P. syringae* pv. *tomato* in a New York state tomato field in 2009 reinforced the need for diagnostic tools for use in quickly discerning the lineage of *P. syringae* strains. To assess the relationship of the new isolate to previously sequenced strains, a draft genome sequences was generated. MLST analysis placed the new isolate in the T1-like subgroup of *P. syringae* pv. *tomato*.

P. syringae virulence is determined by a combination of genetic factors including phytotoxins, T3Es, and the extent to which conserved pattern associated molecular patterns (PAMPs) are detected by host receptors. *P. syringae* T3Es act chiefly by suppressing host immune responses, and are regulated by the HrpL alternative sigma factor (7,32), bioinformatic prediction of which is an efficient strategy for locating T3E genes and other members of the HrpL regulon.

The best characterized representatives of the T1-like and DC3000/*P. syringae* pv. *maculicola* subgroups are T1 and DC3000. The T3E repertoires of these two strains differ significantly, with only 14 T3E genes shared between them and 15 and 11 unique to DC3000 and T1, respectively (2). The repertoire of previously confirmed T3Es and

non-T3E members of the HrpL regulon in NYS-T1 were identified by sequence similarity and HrpL binding site prediction, and were found to be identical to those in T1. NYS-T1 was found to encode a predicted protein with limited similarity to the T3E HopAO1 and downstream of a HrpL binding site. This gene is absent from T1 and DC3000 but its potential role in NYS-T1 virulence remains to be evaluated.

Of the observed differences in predicted virulence factors, the presence of coronatine in NYS-T1 is most significant. Coronatine has been shown to enhance fitness of *P. syringae* pathovars by facilitating entry into plants through stomata and promoting growth in planta (43), and is typically identifiable by the presence of yellowish halos around the speck lesions. Many *P. syringae* pathovars produce coronatine, but the presence of coronatine biosynthetic genes is not closely correlated with strain phylogeny or host range among and within pathovars (5).

The sequence difference in the flgII-28 epitope of the flagellin structural protein (FliC) is also significant given the importance of PAMP-triggered immunity in host defense (9) wherein the host plant recognizes molecular patterns in highly conserved gene products. Cai et al. demonstrated that the flgII-28 sequence in strain Max13 triggers a weaker defense response than the flgII-28 sequence of strains DC3000 or T1 (10). Comparison of FliC sequences among the strains reveals that NYS-T1 encodes the sequence variant present in Max13, potentially contributing to the high virulence of NYS-T1 by reducing the host defense response.

Comparative virulence analyses of *P. syringae* strains in the T1-like and DC3000/*P. syringae* pv. *maculicola* subgroups have consistently shown the former to be the more virulent on tomato. To develop a diagnostic test capable of distinguishing these two subgroups, genome sequences of high virulence T1-like strains were compared with the DC3000 sequence and a core set of confirmed T3Es in the T1-like subgroup identified. Primers were developed from conserved sequences in the subgroup-specific effectors and shown to be effective in discriminating among isolates. Specifically, primers designed against conserved regions in *avrA* and *hopW1* proved diagnostic for the T1-like subgroup and primers against *hopN1* for the DC3000/*P. syringae* pv. *maculicola* subgroup, with *hopR1* discriminating Group I *P. syringae* strains from other phylogenetic *P. syringae* groups as well as other bacteria associated with tomato. Using this protocol, six previously uncharacterized *P. syringae* isolates from the Smart lab were shown to be members of the T1-like subgroup (Table 5, Fig. 2).

The extent of host plant damage in the field of isolation suggested that NYS-T1 exhibited virulence levels equal to or greater than other T1-like strains. Consistent with previous reports (10,31), T1-like strains were the most virulent. Strain T1 itself caused numerically fewer specks per leaflet than other strains in the subgroup, possibly reflecting the absence of coronatine production; however, this difference was not statistically significant (Table 6).

Why the T1-like subgroup exhibits higher levels of virulence on tomato than the DC3000/*P. syringae* pv. *maculicola* strains remains unclear, though differences in the T3E repertoires are suggestive of different “strategies” for pathogenesis. One possibility is that the composition and large number of T3Es encoded by strain DC3000 is necessary for a host range that includes both tomato and brassica species, while the particular complement of T3Es conserved in the T1-like subgroup achieves greater virulence on tomato only. In contrast, neither the genes for coronatine biosynthesis nor the *fliC* allele associated with evasion of innate immunity are strongly associated with subgroup or host range.

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Table 6. Average specks per leaflet on tomatoes inoculated with pseudomonads^z

Isolate	Average specks per leaflet (SE)	
<i>Pseudomonas syringae</i> pv. <i>tomato</i> NYS-T1	7.16 (1.48)	a
<i>P. syringae</i> pv. <i>tomato</i> K40	6.76 (1.02)	a
<i>P. syringae</i> pv. <i>tomato</i> 09084	5.85 (0.67)	a
<i>P. syringae</i> pv. <i>tomato</i> A9	4.76 (0.44)	ab
<i>P. syringae</i> pv. <i>tomato</i> T1	4.71 (0.55)	ab
<i>P. syringae</i> pv. <i>tomato</i> DC3000	1.61 (0.13)	bc
<i>P. viridiflava</i> 10078	0.16 (0.54)	c
Water	0 (0)	c

^z Different letters represent significantly different groups. Numbers reflect average value of two biological repetitions using data 12 days postinoculation.

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