Molecular and Evolutionary Analyses of *Pseudomonas syringae* pv. *tomato* Race 1

Suparat Kunkeaw, Steven Tan, and Gitta Coaker

Department of Plant Pathology, University of California–Davis, Davis 95616, U.S.A.

Submitted 4 August 2009. Accepted 13 November 2009.

Pseudomonas syringae pv. *tomato*, the causal agent of bacterial speck of tomato, has recently become an increasing problem in California due to the evolution and prevalence of highly aggressive race 1 strains. In resistant plant genotypes, the type III effectors AvrPto and AvrPtoB are recognized by the tomato proteins Pto and Prf. We investigated the prevalence of *avrPto* and *avrPtoB* in strains collected over the last 13 years in California. All race 1 strains retained *avrPtoB* but did not express AvrPtoB protein at detectable levels in vitro. However, deletion of *avrPtoB* indicated that this effector protein is still expressed at low levels in race 1 during infection. *avrPto* was detected in four race 1 strains but a key amino acid polymorphism prevents this new protein from interacting with and eliciting Pto-mediated resistance. Growth curve analyses demonstrate that this new *avrPto* allele is still functional and can enhance *P. syringae* virulence on tomato. Multilocus sequence typing was used to resolve phylogenetic relationships and revealed that the majority of race 0 and 1 strains were most closely related to *P. syringae* T1. Collectively, these data support the hypothesis that existing *P. syringae* populations evolved to overcome genetic resistance by altering the expression and sequence of *avrPto* and *avrPtoB* effectors.

In order to successfully avoid infection, plants have evolved a series of defense mechanisms that work in concert to limit pathogen invasion and multiplication (Chisholm et al. 2006). Conceptually, there are two primary branches of plant innate immunity. One branch employs extracellular receptors to recognize conserved microbial features, termed pathogen-associated molecular patterns (PAMPs), resulting in PAMP-triggered immunity (PTI) (Chisholm et al. 2006; Jones and Dangl 2006). The second branch uses intracellular plant resistance (R) proteins to recognize pathogen effectors delivered inside host cells during infection, resulting in effector-triggered immunity (ETI) (Chisholm et al. 2006; Jones and Dangl 2006). Bacterial effectors play dual roles in susceptible and resistant genotypes. In the absence of a corresponding R gene, effectors enhance pathogen virulence and are collectively required for pathogenicity (McCann and Guttman 2008). In the presence of a

corresponding R gene, a particular effector can render the pathogen avirulent. Many type III effectors have been shown to inhibit the function of specific host protein targets which are involved in mediating PTI and ETI defense responses (Zhou and Chai 2008).

Microbial pathogenicity in plants has evolved through an arms race at the molecular level. As microbial pathogens evolve new means to evade defense responses, plants are hypothesized to undergo adaptive evolution, resulting in a continuous battle between plant and pathogen. Insight into how microbial pathogens can evolve to overcome plant innate immunity has come from investigating the molecular basis of resistance breakdown. Studies in bacteria and fungi have demonstrated that pathogen effectors can evade ETI-based resistance through mutations and insertions in the recognized effector, large deletions, and DNA shuffling (Arnold et al. 2007). Wichmann and associates (2005) examined the genetic diversity of the *avrBs2* effector from *Xanthomonas campestris*, which is recognized by the *Bs2* R gene. Mutations in *avrBs2* included point mutations, frameshifts, and large transposon insertions. A second study analyzed the sequence and function of *avrBs2* from *X. campestris* isolated on *Bs2* pepper plants (Gassmann et al. 2000). Four mutant *avrBs2* alleles were identified, which possessed small insertions, deletions, and point mutations. Interestingly, *avrBs2* alleles with point mutations still retained their virulence function but only elicited weak recognition by *Bs2* (Gassmann et al. 2000). Large plasmid deletions were also detected surrounding the *Pseudomonas syringae* pv. *phaseolicola* *avrPphF* effector, which is recognized by the corresponding *R1* gene in bean (Rivas et al. 2005). Alterations in AvrXa7's effector gene structure through terminal reassortment or domain swapping can also result in the generation of new specificities (Yang et al. 2005). Taken together, these studies highlight the diverse mechanisms microbial pathogens can employ to evade ETI.

P. syringae pv. *tomato*, the causal agent of bacterial speck of tomato, is one of the most persistent bacterial diseases found in the tomato-growing regions of North America (Goode and Sasser 1980). *P. syringae* DC3000 can also infect the model plant *Arabidopsis*, and this pathosystem is widely used to investigate the molecular mechanisms controlling plant innate immunity. In the early 1990s, the tomato *Pto* gene (for resistance against *P. syringae* pv. *tomato*) was cloned, which conferred resistance to race 0 (Martin et al. 1993). *Pto* works in concert with another tomato protein, Prf, to control resistance against bacterial speck (Salmeron et al. 1996). *Pto* was first introgressed from *Solanum pimpinellifolium*, a wild tomato species, into tomato cultivars and provided effective disease control for several decades (Pitblado and Kerr 1979, 1980). The unrelated effector proteins AvrPto and AvrPtoB (HopAB2) from *P. syringae* DC3000 induce ETI in resistant tomato geno-

Corresponding author: G. Coaker; Telephone (530) 752-6541; Fax: (530) 752-5674; E-mail: glcoaker@ucdavis.edu

S. Kunkeaw and G. Coaker contributed equally to this work.

Current address of S. Kunkeaw: Institute of Molecular Biosciences, Mahidol University, Thailand.

*The e-Xtra logo stands for “electronic extra” and indicates that Figure 6 appears in color online.

types (Scofield et al. 1996; Mucyn et al. 2006). Recognition of AvrPto and AvrPtoB culminates in a form of programmed cell death at the site of infection, termed the hypersensitive response (HR), and inhibition of pathogen growth. Pto is a Ser/Thr kinase that directly interacts with both effectors (Scofield et al. 1996; Kim et al. 2002).

Both *avrPto* and *avrPtoB* contribute significantly to pathogen virulence, which can explain the historical success of the tomato gene *Pto* in controlling bacterial speck. Deleting either *avrPto* or *avrPtoB* from *P. syringae* DC3000 results in a subtle decrease in virulence, while deleting both genes significantly reduces the pathogenicity of this strain (Lin and Martin 2005). Insights into how *avrPto* and *avrPtoB* act to enhance bacterial virulence by disrupting PTI have been recently discovered in *Arabidopsis*. AvrPto and AvrPtoB target the receptor-like kinase BAK1, which serves as a signaling partner with both PAMP and brassinosteroid pattern-recognition receptors (Shan et al. 2008). AvrPto and AvrPtoB can also associate with the PAMP receptor FLS2, which acts in concert with BAK1 to detect the presence of the bacterial PAMP flagellin (Shan et al. 2008; Xiang et al. 2008). AvrPto acts as a kinase inhibitor and can inhibit the Pto kinase in vitro (Xing et al. 2007). AvrPtoB's N-terminal domain is required for ETI; its C-terminus is an E3 ubiquitin ligase and is required for its virulence and anti-programmed cell death functions (Abramovitch et al. 2003, 2006; Janjusevic et al. 2006). Recently, it has been shown that AvrPtoB ubiquitinates FLS2, targeting it for degradation (Göhre et al. 2008). Taken together, these results highlight the importance of AvrPto and AvrPtoB in inhibiting PTI through their PAMP receptor and co-receptor targets.

The purpose of this study was to investigate the molecular basis of resistance breakdown in *P. syringae* strains collected in Northern California over the last decade. Our results indicate that current *P. syringae* strains obtained from infected tomato plants are all race 1 and can defeat *Pto*-mediated resistance, and most exhibit increased virulence compared with laboratory strains. Multilocus sequence typing (MLST) has revealed that the majority of strains are very closely related *P. syringae* T1 and possess identical sequence types. We also investigated the function and prevalence of the *avrPto* and *avrPtoB* effectors. Collectively, these data indicate that the *P. syringae* population in California has evolved to overcome *Pto*-mediated resistance by altering the expression and sequence of

the AvrPto and AvrPtoB effectors as well as acquiring novel virulence factors.

RESULTS

Recent *P. syringae* strains isolated in California are race 1 and exhibit enhanced virulence on tomato.

In order to gain a more complete understanding of the genetic and phenotypic diversity of a collection of *P. syringae* pv. *tomato* strains, we investigated the population structure and virulence of strains collected throughout California over the last 13 years. The first report of race 1 strains detected in California occurred with the identification of *P. syringae* A9, collected in 1996 (Table 1) (Arredondo and Davis 2000). In 2005, there was an outbreak of bacterial speck of tomato in California and the vast majority of strains collected from 2005 to 2007 were race 1 (M. Davis, *personal communication*). We were unable to identify any race 0 strains in 2007, 2008, or 2009 from infected tomato plants in the field. Individual colonies were isolated from infected tomato leaves and tested for fluorescence on King's B (KB) media. Purified strains were then tested for their virulence and infectivity on the tomato line Rio-Grande 76R (*Pto/Pto Prf/Prf*) and the Rio-Grande 76R *pto* mutant (*pto11/pto11 Prf/Prf*) (Table 1). Race 0 strains, such as *P. syringae* DC3000, are recognized by *Pto* in Rio-Grande 76R and can only cause disease on Rio-Grande 76R *pto11*. In contrast, race 1 strains can avoid *Pto*-mediated resistance and cause disease on both Rio-Grande 76R and 76R *pto11*. We found that 16 of the 17 race 1 strains were as virulent or more virulent than *P. syringae* T1, a well-characterized race 1 strain frequently used in the laboratory, by visual analysis of disease symptoms (Table 1) (Almeida et al. 2009). Growth curve analyses on Rio-Grande 76R demonstrated that multiple California strains can grow to significantly higher levels (8- to 13-fold) than *P. syringae* T1 and cause more disease symptoms (Fig. 1A and B). We also investigated bacterial aggressiveness on the processing cv. AB 2. AB 2 is currently the most popular processing cultivar in California, accounting for 15.4% of processing loads in 2008 (California Processing Tomato Advisory Board). *P. syringae* strains A9, 838-16, and 19 all displayed enhanced disease symptoms on AB 2, indicating that these strains are also hypervirulent on common tomato cultivars (Fig. 1C).

Table 1. *Pseudomonas syringae* strains isolated in California differ in virulence and the presence of individual effectors^a

Strain	County isolated	Year	<i>avrPto</i>	<i>avrPtoB</i>	Virulence, tomato	Virulence, <i>Arabidopsis</i>	Coronatine production
315	San Benito	1998	–	+	nt	nt	nt
316	Yolo	1998	+	+	nt	nt	nt
A9	Yolo	1996	–	+	Hypervirulent	–	++
407	Sacramento	1997	–	+	Virulent	–	+
838-16	Yolo	2005	+	+	Hypervirulent	–	+
836-2	Yolo	2005	–	+	Hypervirulent	–	++
838-6	San Joaquin	2005	–	+	Virulent	–	++
838-8	San Joaquin	2005	+	+	Hypervirulent	–	++
838-9	San Joaquin	2005	–	+	Virulent	–	+
838-4	Colusa	2005	–	+	Hypervirulent	–	+++
16	Yolo	2008	–	+	Hypervirulent	–	+
24	Yolo	2008	+	+	Virulent	–	+
410	Yolo	2008	–	+	Hypervirulent	–	–
22	Sacramento	2008	+	+	Hypervirulent	–	+++
23	Sacramento	2008	–	+	Virulent	–	+
20	Kern	2008	–	+	Virulent	–	–
21	Kern	2008	–	+	Hypervirulent	–	–
18	Kern	2008	–	+	Weakly virulent	–	–
19	Kern	2008	–	+	Hypervirulent	–	++

^a Virulence was determined by bacterial growth curves and visually inspecting disease symptoms following bacterial inoculation. Strains 315 and 316 are race 0. All other strains are race 1 and their virulence was compared with that of *P. syringae* T1 on *Solanum lycopersicum* cvs. Rio-Grande 76R and the *pto* mutant 76R *pto11*. All strains tested were not able to infect *Arabidopsis rps2-101c/rpm1-3*; nt = not tested.

We also investigated individual strains for their ability to infect *Arabidopsis* and produce the toxin coronatine. Surprisingly, we found that coronatine production differed among strains and the ability to produce coronatine did not correlate with enhanced virulence on tomato (Table 1). We used *P. syringae* DC3000 as a positive control for coronatine production. The coronatine mutant, *P. syringae* DC3000 *cor*⁻ (Sreedharan et al. 2006), and *P. syringae* T1 were used as negative controls in our coronatine production assay. Because coronatine is an important phytotoxin involved in overcoming the plant innate immune system (Nomura et al. 2005; Sreedharan et al. 2006), these results suggest that certain strains have most likely acquired novel virulence factors, including toxins. We also found that none of the race 1 strains could induce disease symptoms in *Arabidopsis* after syringe infiltration (Table 1). We used the *Arabidopsis* Col-0 *rps2-101c/rpm1-3* mutant for infection assays in order to decrease the chance of ETI-mediated recognition. Growth curve analyses demonstrated that the strains *P. syringae* A9, 838-16, and 19 could not cause disease on *Arabidopsis* (Fig. 1D and E). To date, only *P. syringae* DC3000 can infect *Arabidopsis*, while all other tested *Pseudomonas* strains isolated from tomato cannot (Yan et al. 2008; Almeida et al. 2009).

Phylogenetic characterization of *P. syringae* strains isolated in California.

In order to investigate the relationship between *P. syringae* strains isolated in California and other closely related *P. syringae* pathovars, we performed MLST, a typing method for the characterization of bacterial strains by examining the nucleotide sequence of multiple housekeeping genes, or fragments of them, for evolutionary and population genetics analyses (Maiden et al. 1998). We sequenced portions of seven core genome genes that have been previously demonstrated to be useful in elucidating *P. syringae* phylogeny (*gap1* *rpoD* *pgi* *gyrB* *kup* *acnB*, and *gltA*) (Sarkar and Guttman 2004; Yan et al. 2008). We included the gene sequences of closely related *P. syringae* strains previously described by Yan and colleagues in our analyses (Yan et al. 2008). All strains belonging to each sequence type are listed in Table 2. The sequences of the seven genes of each sequence type were concatenated and a maximum likelihood tree and a neighbor-joining tree were constructed and rooted with the sequence of *P. syringae* pv. *syringae* B728A (Fig. 2). The neighbor-joining tree (Fig. 2) and the maximum likelihood tree (not shown) had the same overall topology and possessed similar branch lengths.

Of the 17 race 1 California strains analyzed, 16 possessed identical sequence types. Phylogenetic analyses indicate that these 16 California strains possessed the same sequence type as *P. syringae* T1 (Table 2; Fig. 2). The majority of *P. syringae* strains characterized to date possess identical sequence types

with either *P. syringae* T1 or *P. syringae* JL1065 (Yan et al. 2008). Interestingly, *P. syringae* 18 was most closely related to strains of *P. syringae* pv. *apii* (Pap), which were isolated from celery. Previously, Yan and colleagues (2008) found that Pap1089 was unable to infect the *S. lycopersicum* cv. SunPride based on visual inspection of disease symptoms. However, some *P. syringae* pv. *apii* can infect both celery and tomato (B. Vinatzer, *personal communication*). Although *P. syringae* 18 is the least virulent of the strains collected in California, we were still able to detect bacterial speck disease symptoms on the tomato genotypes Rio-Grande 76R and 76R *pto11*. Growth

Fig. 1. *Pseudomonas syringae* pv. *tomato* strains isolated in California are race 1 and are hypervirulent compared with *P. syringae* T1. **A**, Four-week-old Rio Grande 76R tomato plants were dip inoculated with different *P. syringae* strains and subjected to growth curves 0 and 4 days post inoculation. Dip inoculations were performed at a concentration of 1×10^7 CFU/ml. Results are shown as the mean ($n = 4$), including standard deviation. Statistical differences were detected by Fisher's least significant difference, $\alpha = 0.05$ (Snedecor and Cochran 1980). **B**, Disease symptoms 4 days post inoculation on Rio Grande 76R. **C**, Disease symptoms 4 days post inoculation on the processing cv. AB 2. **D**, *P. syringae* strains cannot infect *Arabidopsis*. Four-week-old *Arabidopsis* *rps2-101c/rpm1-3* plants were inoculated by syringe infiltration with different *P. syringae* strains. Syringe inoculations were performed at a concentration of 0.5×10^5 CFU/ml. Results are shown as the mean ($n = 4$), including standard deviation. Statistical differences were detected with a two-tailed *t* test ($\alpha = 0.001$). **E**, *Arabidopsis* disease symptoms 4 days post inoculation.

curve analyses with *P. syringae* 18 were also performed on Rio-Grande 76R and 76R *pto11*. *P. syringae* 18 is able to grow approximately 1.7 log in both tomato genotypes, indicating that this strain can, indeed, infect tomato and is race 1 (data not shown).

We also included two *P. syringae* race 0 strains, 315 and 316, in our MLST analyses (Table 1; Fig. 2). Interestingly, both strains were closely related to *P. syringae* T1. *P. syringae* 316 possessed a sequence type identical to T1, while strain 315 differed by a single nucleotide polymorphism in the *pgi* allele (Fig. 2). The observation that both *P. syringae* strains 315 and 316 possess MLST profiles similar to all other California strains suggests that race 1 strains either recently evolved from existing *P. syringae* populations due to widespread cultivation of cultivars possessing *Pto* or have been historically present at low levels in the pathogen population.

***P. syringae* strains isolated in California have evolved to overcome Pto-mediated resistance in tomato by altering the expression, sequence, and prevalence of AvrPto and AvrPtoB.**

Previous reports have shown that *avrPto* and *avrPtoB* contribute significantly to *Pto* virulence on susceptible tomato cultivars (Lin and Martin 2005). The majority of the California race 1 strains tested were more virulent, growing 8- to 13-fold more than previously characterized *P. syringae* strains. In order to investigate the prevalence and function of *avrPto* and *avrPtoB* in this population, we sequenced and analyzed the expression pattern of both effectors. We found that 4 of the 17 race 1 *P. syringae* strains possessed *avrPto* (Table 1). The *avrPto* genes in the California race 1 strains were identical and possessed five amino acid polymorphisms when compared with the DC3000 allele. Fifteen race 1 *avrPtoB* homologs were nearly identical to *avrPtoB* from T1 (*AvrPtoB_{T1}*), with only one polymorphism that changed that amino acid at position 6 from a glycine to an arginine. These effector homologs will be referred to as *avrPto₈₃₈₋₁₆* and *avrPtoB₁₉* throughout the remainder of this article and were named after the strain from which they were first identified (Table 1). We also sequenced one *avrPtoB* homolog from strain 18 that was related to a partial sequence of *avrPtoB* from *P. syringae* pv. *thea*, the causal agent of black rot of tea. However, *avrPtoB₁₈* possessed a single base pair insertion near the 5' end of the gene, rendering it nonfunctional.

Next, we investigated whether *avrPto₈₃₈₋₁₆* and *avrPtoB₁₉* are expressed. Culturing gram-negative plant-pathogenic bacteria

at low temperature and low pH in minimal media mimics infection conditions and induces effector expression as well as secretion (Huynh et al. 1989). *P. syringae* strains were cultured in minimal media and separated into cell and supernatant fractions. The cellular fraction was used to analyze *avrPto* and *avrPtoB* expression at the RNA and protein level. We were able to detect the expression of both homologs by reverse-transcriptase polymerase chain reaction (RT-PCR), indicating that these are functional genes whose expression is most likely induced during infection (Fig. 3A). *AvrPto₈₃₈₋₁₆* is also expressed and secreted by the T3SS (Fig. 3B). We could not detect protein-level expression of *AvrPtoB₁₉* or *AvrPtoB_{T1}* (Fig. 3B). Previously, Lin and Martin (2007) showed that *avrPtoB_{T1}* was expressed at the RNA level but the protein did not accumulate in *P. syringae* T1 when grown in minimal media (Lin and Martin 2007). These results demonstrate that modulating *AvrPtoB* expression is a common mechanism used by multiple race 1 strains to overcome *Pto*-mediated resistance.

Although we were unable to detect *AvrPtoB₁₉* protein expression by Western blot under laboratory conditions, it is still

Fig. 2. Neighbor-joining tree of concatenated DNA fragments. Representative strains for each sequence type (ST) are shown and the number of strains belonging to each ST is indicated in parentheses. *Pseudomonas syringae* pv. *syringae* B728A (*PsyB728A*) was used as the outgroup. *Pto* = *P. syringae* pv. *tomato*, *Pan* = *P. syringae* pv. *antirrhini*, *Pma* = *P. syringae* pv. *maculicola*, and *Psy* = *P. syringae* pv. *syringae*.

Table 2. Sequence types (ST) of strains used in this study^a

ST number	Isolates
1	Pan126, Pan152E, Pan4303
3	PtoT1, PtoMax1, PtoMax13, PtoPST6, PtoPT13, PtoPT14, PtoPT18, PtoPT2, PtoPT21, PtoPT26, PtoPT32, PtoNCPP1108, PtoB181, Pto1318, Pto487, PtoKS127, Pto838-4, Pto838-6, Pto838-8, Pto838-9, Pto838-16, Pto836-2, PtoA9, Pto407, Pto410, Pto16, Pto19, Pto20, Pto21, Pto22, Pto23, Pto24
4	PtoMax14
5	PtoJL065, PtoJL1031, PtoPT28, PtoPT29, PtoPT30, PtoPST26L, PtoKS112
6	PmaF1, PmaF7
7	Pap1089, Pto18
8	PmaF15
9	PmaM3
10	PmaM6, PmaM1, PmaM2, PmaM8, Pma1766
11	PtoICMP3443
12	PtoICMP3449
13	PtoDC3000, PtoOH314
14	PmaF9, PmaF6, PmaF10A, PmaF16, PmaF17, PmaF18, PmaF19, Pma84-59, PmaICMP3435, PmaICMP3455, PmaICMP9305
15	Pto315
16	PsyB728a

^a All other *Pseudomonas syringae* strains and ST have been described previously (Yan et al. 2008). *Pto* = *P. syringae* pv. *tomato*, *Pan* = *P. syringae* pv. *antirrhini*, *Pma* = *P. syringae* pv. *maculicola*, *Pap* = *P. syringae* pv. *apii*, and *Psy* = *P. syringae* pv. *syringae*.

possible that AvrPtoB₁₉ proteins are weakly expressed during infection and contribute to pathogen virulence or avirulence. After conducting plant inoculations with multiple race 1 strains, we observed that those strains grew four- to sevenfold more on the Rio-Grande 76R *pto11* genotype than 76R, although these two tomato cultivars are near-isogenic lines (Fig. 4A). This observation supports the hypothesis that AvrPtoB₁₉ protein may still be expressed at a low level during infection. In order to test this hypothesis, two independent *P. syringae* 19 Δ *avrptob* knockout mutants were generated and subjected to growth curve analyses on Rio-Grande 76R and 76R *pto11*. *P. syringae* 19 Δ *avrptob* grows to equal levels on 76R and 76R *pto11*, whereas wild-type *P. syringae* 19 exhibited more growth on 76R *pto11* than 76R (Fig. 4). These results suggest that a small amount of the AvrPtoB effector is delivered into plant cells by race 1 strains. We were also able to detect a decrease in virulence of 1.5- to 4-fold on *P. syringae* 19 Δ *avrptob* knockouts when compared with *P. syringae* 19 inoculated on Rio Grande 76R *pto11* across multiple experiments. However, the statistical significance of this trend varied from experiment to experiment.

Our analysis of different race 1 strains collected in California revealed that four race 1 strains possess AvrPto₈₃₈₋₁₆, a novel homolog that is expressed and secreted via the T3SS (Table 2; Fig. 4). There are five amino acid polymorphisms between AvrPto_{DC3000} and AvrPto₈₃₈₋₁₆ (Fig. 5A). Previously, AvrPto_{DC3000} was shown to interact with tomato Pto by yeast two-hybrid analysis (Scofield et al. 1996; Tang et al. 1996). The co-crystal structure of AvrPto_{DC3000} in complex with Pto has also been published (Xing et al. 2007). This structural analysis revealed that AvrPto_{DC3000} directly interacts with Pto at two interfaces: i) one end of an AvrPto helical bundle (Y89, M90, L101, and P102) with one Pto loop preceding β 1, and ii) the AvrPto GINP motif with the Pto P+1 loop (Xing et al. 2007). AvrPto₈₃₈₋₁₆ contains a point mutation in its GINP loop (G95E) when compared with AvrPto_{DC3000} (Fig. 5A). This region of the protein mediates contact with Pto and is required for Pto-mediated resistance in DC3000 (Wulf et al. 2004; Xing et al. 2007). We hypothesized that AvrPto₈₃₈₋₁₆ is no longer recognized by Pto due to the G95E substitution. In support of this hypothesis, the AvrPto allele from race 0 strain 316 possesses a glycine at position 95 but retains all four other polymorphisms present in AvrPto₈₃₈₋₁₆ (Fig. 5A). In order to investigate whether AvrPto₈₃₈₋₁₆ is still able to interact with Pto, we performed yeast two-hybrid analysis. As expected, we were able to detect a robust interaction between Pto, AvrPto_{DC3000}, and the AvrPto₈₃₈₋₁₆ (E95G) mutant by yeast two-hybrid analysis. However, we were not able to detect an interaction between Pto and AvrPto₈₃₈₋₁₆

by yeast two-hybrid analysis, indicating that Pto cannot interact with AvrPto₈₃₈₋₁₆ due to a mutation in its GINP loop (Fig. 5D). All AvrPto alleles are expressed at similar levels in yeast (Fig. 5E).

We also investigated whether AvrPto₈₃₈₋₁₆ could be recognized by Pto and Prf when expressed using *Agrobacterium*-mediated transient expression. As expected, transient expression of AvrPto_{DC3000} resulted in a robust hypersensitive response (HR) 30 h post infiltration on Rio Grande 76R but not 76R *pto11* (Fig. 5B). Transient expression of AvrPto₈₃₈₋₁₆ did not induce an HR in Rio Grande 76R but transient expression of

Fig. 4. *Pseudomonas syringae* 19 Δ *avrptob* knockout grows to similar levels on Rio-Grande 76R and 76R *pto11*. **A**, *P. syringae* race 1 strains are less virulent on tomato genotypes possessing Pto-mediated resistance. Four-week-old Rio-Grande 76R and 76R *pto11* plants were dip inoculated and growth curves conducted 4 days post inoculation. Results are shown as the mean ($n = 4$), including standard deviation. **B**, *P. syringae* 19 Δ *avrptob* knockout grows to similar levels on Rio-Grande 76R and 76R *pto11*. Results from two independent knockout lines are shown. Dip inoculations were performed at a concentration of 1×10^7 CFU/ml. Results are shown as the mean ($n = 4$), including standard deviation. Statistical differences were detected by Fisher's least significant difference, $\alpha = 0.05$ (Snedecor and Cochran 1980).

Fig. 3. Expression of AvrPto and AvrPtoB in *Pseudomonas syringae* strains by reverse-transcriptase polymerase chain reaction (RT-PCR) and immunoblot. Bacteria were grown in rich (NYG) or minimal (HMM) media. RNA was extracted from bacterial pellets. Protein extracts were analyzed from bacterial pellets (P) and culture supernatant (S). **A**, *avrPtoB* and *avrPto* alleles are expressed at the RNA level in California race 1 strains. RNA was isolated and subjected to RT-PCR. *AvrPtoB* primers were designed to only detect race 1 alleles (T1 and 19). **B**, AvrPto protein is expressed in California race 1 strains and AvrPto from isolate 838-16 is secreted by the type III secretion system. However, AvrPtoB protein expression is not detectable in race 1 strains. Proteins were subjected to an anti-AvrPto, anti-AvrPtoB, and anti-NPTII immunoblots. NPTII is a soluble, unsecreted protein.

AvrPto₈₃₈₋₁₆ (E95G) did induce an HR (Fig. 5B). Western blots were conducted to verify that both genes were expressed using transient expression in *Nicotiana benthamiana*. *N. benthamiana* was used to detect protein expression because infiltration of tomato with *Agrobacterium* species at an optical density at 600 nm (OD₆₀₀) over 0.1 induced an HR due to PTI, making it difficult to detect transient protein expression by Western blot in tomato. We were able to detect expression of all AvrPto alleles (Fig. 5C). Taken together, these results indicate that AvrPto₈₃₈₋₁₆ can no longer interact with Pto due to a single amino acid change in the GINP loop to EINP and, as a result, can no longer elicit Pto-mediated resistance.

In order to determine whether *avrPto*₈₃₈₋₁₆ can still confer a virulence advantage, we expressed this effector in *P. syringae*

19 *avrptob-1* and conducted growth curves on tomato Rio Grande 76R *ptol1*. Both *avrPto*₈₃₈₋₁₆ and *avrPto*_{DC3000} enhanced *P. syringae* 19 *avrptob-1* virulence (Fig. 6A and B). *P. syringae* 19 *avrptob-1* expressing either *avrPto* allele grew sevenfold more on Rio Grande 76R *ptol1* and exhibited more severe disease symptoms. These results indicate that *avrPto*₈₃₈₋₁₆ can still promote bacterial virulence on tomato.

DISCUSSION

How has the *P. syringae* population in California evolved to overcome Pto-mediated resistance in tomato? For over 20 years, genetic resistance conferred by the tomato genes *Pto* and *Prf* has been deployed to help control bacterial speck disease pressure

Fig. 5. A novel *avrPto* allele present in *Pseudomonas syringae* race 1 isolates is no longer recognized in tomato. **A**, Clustal W alignment of AvrPto from *P. syringae* DC3000 (race 0), *P. syringae* 316 (race 0), and *P. syringae* 838-16 (race 1). **B**, AvrPto₈₃₈₋₁₆ is no longer recognized by Pto and does not elicit a Pto-dependent hypersensitive response in tomato due to the presence of a glutamic acid (E) residue at position 95. *Agrobacterium* spp.-mediated transient expression of AvrPto:HA_{DC3000}, AvrPto:HA₈₃₈₋₁₆, and AvrPto:HA₈₃₈₋₁₆ (E95G) in Rio-Grande 76R and 76R *ptol1* plants. *Agrobacterium* sp. was infiltrated at an optical density at 600 nm (OD₆₀₀) = 0.1 and the picture taken 24 h post infiltration; 1 = pMD1 GFP, 2 = AvrPto:HA_{DC3000}, 3 = pMD1 AvrPto:HA₈₃₈₋₁₆, 4 = AvrPto:HA₈₃₈₋₁₆ (E95G). **C**, Western blot verifying expression of AvrPto:HA alleles in *Nicotiana benthamiana*. *Agrobacterium* sp. was infiltrated at an OD₆₀₀ = 1.0 and samples were taken 40 h post infiltration. **D**, AvrPto₈₃₈₋₁₆ no longer interacts with *Solanum pimpinellifolium* Pto by yeast two-hybrid analysis. EV = empty vector; pGAD = pGADT7 activation domain vector; pGBD = pGBDKT7 DNA binding domain vector; SD-2 = synthetic dextrose media lacking leucine and tryptophan; and SD-3 = synthetic dextrose media lacking leucine, histidine, and tryptophan. **E**, Anti-hemagglutinin Western blot demonstrating that both AvrPto alleles are expressed in yeast.

(Pedley and Martin 2003). In California, *P. syringae* infections are primarily confined to the Northern region of the state and disease pressure increases during cool, wet springs. The vast majority of tomato crops grown in California are field-grown processing (canning) tomato cultivars. The top 50 processing cultivars comprise 98% of the total cultivars planted in California (California Processing Tomato Advisory Board). In 2008, 90% of the harvested tomato loads were from processing cultivars possessing genetic resistance to *P. syringae* race 0 (California Processing Tomato Advisory Board). The widespread use of cultivars recognizing the *avrPto* and *avrPtoB* effectors present in race 0 strains may provide strong selective pressure to overcome *Pto*-mediated resistance within *P. syringae* bacterial populations. Other microbial pathogens have been shown to overcome race-specific resistance by effector deletions, rearrangements, and mutations (Arnold et al. 2007).

We investigated the prevalence and function of both *avrPto* and *avrPtoB* within California strains. We were able to identify a novel *avrPto* allele, *avrPto*₈₃₈₋₁₆, that is present, expressed, and secreted in four strains (Fig. 3). AvrPto₈₃₈₋₁₆ could not interact with tomato *Pto* by yeast two-hybrid analysis or elicit *Pto*-mediated resistance, because of a key amino acid polymorphism in the GINP loop where the glycine residue present in the DC3000 allele corresponds to a glutamic acid residue present in the 838-16 allele (Fig. 5A). This region has been shown to make direct contact with *Pto* by structural analyses (Xing et al. 2007). Interestingly, the *avrPto*₃₁₆ allele, which was isolated from a race 0 isolate, retains the GINP loop but is otherwise identical to *avrPto*₈₃₈₋₁₆. A second race 0 isolate did not possess *avrPto* but did possess a T1-like *avrPtoB* allele. Our molecular phylogenetic data indicate that the majority of *P. syringae* strains isolated in California are very closely related and cluster with *P. syringae* T1 (Fig. 2). These results support the hypothesis that race 1 strains recently evolved from existing *P. syringae* populations. In order to overcome *Pto*-mediated resistance, it is likely that all *P. syringae* populations would need to alter their expression of AvrPtoB but only some would either need to lose the *avrPto* gene completely or mutate *avrPto* in order to evade *Pto*-mediated recognition.

Growth curve analyses with *avrPto*₈₃₈₋₁₆ demonstrated that this effector can still function to enhance *P. syringae* virulence (Fig. 6). These data indicate that *P. syringae* was able to alter AvrPto's amino acid sequence to overcome *Pto*-mediated resistance without a commensurate decrease in virulence. Similar results were found in a site-directed mutagenesis screen of the *avrPto* effector, where selected mutations could uncouple virulence and avirulence properties (Shan et al. 2000). These results are also similar to what was found with *X. campestris* *avrBs2* alleles isolated from resistant pepper genotypes. Gassmann and colleagues (2000) found that naturally occurring *avrBs2* alleles with R403P or A410D still retained virulence attributes but were partially compromised in their ability to elicit *Bs2*-mediated resistance. Recently, it has been demonstrated that AvrPto has a second virulence activity, independent of the GINP loop, which is located in the carboxy terminal domain and is phosphorylated in planta (Anderson et al. 2006; Yeam et al. 2009). Phosphorylation of the carboxy terminal domain on S147 and S149 promotes bacterial virulence in an FLS2/BAK1-independent manner (Yeam et al. 2009). Therefore, it is likely that the AvrPto₈₃₈₋₁₆ allele can still promote bacterial virulence through its carboxy terminal domain.

We also investigated the prevalence and expression of the *avrPtoB* effector. AvrPtoB was detected across all *P. syringae* strains (Table 1). DNA sequencing of this locus revealed that it was closely related to *avrPtoB*_{T1}, with only one amino acid polymorphism at the N terminus of the protein. As with *P. syringae* T1, we could only detect RNA but not protein-level expres-

sion of *avrPtoB*₁₉ in California race 1 strains (Fig. 3). Previously, Lin and colleagues (2006) demonstrated that the genetic background of *P. syringae* T1 was responsible for inhibiting *avrPtoB* expression. AvrPtoB_{DC3000} protein does not accumulate when expressed in *P. syringae* T1; AvrPtoB_{T1} protein only accumulates when expressed in *P. syringae* DC3000 (Lin et al. 2006). Taken together, these data indicate that modulating AvrPtoB expression is a key mechanism used to overcome *Pto*-mediated resistance. Our growth curve analyses on *P. syringae* 19 *avrptob* knockouts support the hypothesis that *avrPtoB* is still delivered at a low level into plant cells (Fig. 4). It will be important to determine whether low-level expression of *avrPtoB* in race 1 strains can still inhibit pathogen growth due to weak *Pto*-mediated resistance under field conditions. Exactly how race 1 strains control the protein-level expression of AvrPtoB remains unknown; however, it is a common mechanism shared among *P. syringae* race 1 strains. It is plausible that altering expression of key bacterial effectors is a conserved strategy employed by multiple bacterial pathogens. Work in *P. aeruginosa* has revealed that, although effector genes can be retained across multiple clinical strains, their expression during infection can vary dramatically, and increased levels of effector expression correlate with strain aggressiveness (Roy-Burman et al. 2001; Hauser et al. 2002; Schulert et al. 2003). Our investigation of the molecular basis of resistance breakdown in tomato to *P. syringae* highlights the diversity of approaches bacterial pathogens can use to mask effector recognition in plants.

MATERIALS AND METHODS

Plant materials.

Tomato (*S. lycopersicum*) Rio-Grande (RG) lines were grown in the greenhouse at 24°C, with 14-h days and 60% relative

Fig. 6. *avrPto*₈₃₈₋₁₆ allele contributes to *Pseudomonas syringae* virulence on tomato. **A**, Expression of *avrPto*_{DC3000} or *avrPto*₈₃₈₋₁₆ from the broad host range vector pBAV226 enhances virulence of the *P. syringae* 19 *Δavrptob-1* on tomato. Four-week-old Rio Grande 76R *ptol1* plants were dip inoculated at a concentration of 1×10^7 CFU/ml and growth curves conducted 4 days post inoculation. Results are shown as the mean ($n = 4$), including standard deviation. Statistical differences were detected with a two-tailed *t* test ($\alpha = 0.01$). **B**, Disease symptoms 4 days post inoculation on Rio Grande 76R *ptol1*. **C**, Western blot analyses demonstrating that both AvrPto_{DC3000} and AvrPto₈₃₈₋₁₆ are expressed in *P. syringae*.

humidity. For all experiments, 5- to 6-week-old plants were used. Two genotypes were included: RG-76R (*Pto/Pto Prf/Prf*) and RG-76R *pto11* (*pto11/pto11 Prf/Prf*) (Salmeron et al. 1994).

Arabidopsis Columbia (Col-0) and the *rps2-101c/rpm1-3* mutant (Mindrinos et al. 1994; Boyes et al. 1998) derived from Col-0 were sown in soil and stratified at 4°C for 2 days. Plants were grown in a controlled environment chamber at 24°C with a photoperiod of 10 h of light and 14 h of darkness under a light intensity of 85 µE/m²/s. For all the experiments, 4- to 5-week old plants were used.

Bacterial strains and plant inoculations.

All *P. syringae* strains were grown in KB medium (King et al. 1954) at 30°C. *Escherichia coli* DH5α and DB3.1 were used for plasmid maintenance and grown in Luria broth (LB) (Ham et al. 1998) at 37°C. *Agrobacterium tumefaciens* C58C1 was grown in LB medium at 30°C. The concentrations of antibiotics used in the selective media were as follows: carbenicillin, 50 µg/ml; kanamycin, 25 µg/ml; rifampin, 100 µg/ml; spectinomycin, 50 µg/ml; and tetracycline, 10 µg/ml.

All *P. syringae* strains isolated in California were collected from infected tomato plants grown in the field. Infected tomato leaves were surface sterilized in 70% ethanol for 30 s. Leaf tissue, sampled with a 1-cm hole punch, was ground in 200 µl of 10 mM MgCl₂. Buffer (10 µl) was then used to streak onto KB plates (King et al. 1954). Single fluorescent colonies were tested for their ability to infect the tomato genotypes RG 76R *pto11* and RG 76R as described below. *P. syringae* strains collected in 1996 to 1998 and 2005 were provided by M. Davis (Arredondo and Davis 2000).

P. syringae strains were syringe infiltrated into *Arabidopsis* leaves as described previously (Liu et al. 2009). Tomato genotypes were dip inoculated for 30 s in 10 mM MgCl₂ and 0.008% Silwett L-77 at a concentration of 1 × 10⁷ CFU/ml. For growth curves and secretion assays, *P. syringae* strains carried the pDSK519 broad host range vector to confer kanamycin resistance. The concentration of antibiotics used for growth curves was decreased by half. All plant inoculations were conducted with four biological replicates and repeated at least twice.

Coronatine production.

Coronatine production was measured using a potato tuber assay to detect coronatine-induced hypertrophy as previously described (Coplin and Kado 2001). The *P. syringae* *cor*-mutant (Sreedharan et al. 2006) and *P. syringae* T1 were used as negative controls for coronatine production. *P. syringae* DC3000 was used as a positive control. Coronatine assays were repeated twice with identical results.

MLST.

P. syringae genomic DNA was extracted as previously described (Coplin and Kado 2001) and used to amplify the fragment of housekeeping genes. In total, seven primers (*gap1*, *rpoD*, *pgi*, *gyrB*, *kup*, *acnB*, and *gltA*) were used for MLST and the primer sequences have been described previously (Yan et al. 2008). Gene fragments were amplified from genomic DNA of *P. syringae* strains and gel purified using a GelElute Extraction Kit (5PRIME Inc., Gaithersburg, MD, U.S.A.). Pfu-Turbo DNA polymerase was used for PCR amplification (Stratagene, La Jolla, CA, U.S.A.). DNA sequencing chromatograms were reviewed and edited with Vector NTI (Invitrogen, Carlsbad, CA, U.S.A.). Edited sequences were aligned in Vector NTI and trimmed to generate fragments of identical lengths. We also included the DNA sequences from closely related *P. syringae* strains for our MLST analyses (Yan et al. 2008). Analyses of nucleotide substitution and diversity in the bacterial genes were performed with START2 (Jolley et al.

2001). In order to identify distinct sequence types, a number was assigned to each distinct allele within a locus. Each strain was given seven numbers that represented its strain type. A table was prepared (Table 2) and all the alleles were provided allele numbers according to the Plant Associated and Environmental Microbes database. Strains with an identical allele were given the same number. Identical sequence types have identical alleles across all seven housekeeping genes. Concatenated sequences for each sequence type were generated in START2 and the resulting file used for phylogenetic analysis using MEGA4 (Tamura et al. 2007). We used the neighbor-joining method for phylogenetic analyses with bootstrap analysis (1,000 replications). The tree was rooted with *P. syringae* pv. *syringae* B728A (PsyB728a) as the outgroup.

RT-PCR.

Bacterial cultures were grown for 16 h in 5 ml of *hrp*-inducing liquid minimal media at 17°C as previously described (Mudgett and Staskawicz 1999). Total RNA was extracted from bacterial pellets using the TRIZOL reagent (Invitrogen) and subjected to Dnase I digestion. The first-strand cDNA was synthesized by using 5 µg of total RNA using random primers with a cDNA synthesis kit (Promega Corp.) in a 20-µl reaction. PCR was performed using primers AvrPto F (5'-ATGGG AAATATAGTTGTGTCG) and AvrPto R (5'-TTGCCAGTTACG GTACGG) or AvrPtoB1492 F (5'-GACGACGCGACGCAAT GCCTT) and AvrPtoB R (5'-TCAGGGGACTATTCTAAAA GCA). 16SrRNA was amplified as an internal control using primers 16S F (5'-GCGGCAGGCCTAACACAT) and 16S R (5'-GTTCCCTACGGCTACCTT).

Immunoblotting.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequent immunoblotting were performed according to standard procedures (Harlow and Lane 1988). AvrPto and AvrPtoB immunoblots were performed with affinity purified rabbit polyclonal antisera at a concentration of 1:1,000. Antisera recognizing both proteins were a gift from G. Martin (Lin et al. 2006). NptII immunoblots were performed with rabbit polyclonal antisera at a concentration of 1:1,000 (5' to 3'). Hemagglutinin (HA) immunoblots were performed with mouse monoclonal antisera at a concentration of 1:1,000 (GE Healthcare, Piscataway, NJ, U.S.A.). Secondary goat anti-rabbit or goat anti-mouse IgG-HRP conjugate (Bio-Rad, Hercules, CA, U.S.A.) was used at a concentration of 1:3,000 for detection via enhanced chemiluminescence (Pierce, Rockport, IL, U.S.A.).

For AvrPto and AvrPtoB expression analyses, bacterial cultures were grown for 16 h in 5 ml of *hrp*-inducing liquid minimal media at 17°C (Mudgett and Staskawicz 1999). Bacterial pellets were boiled in 100 µl of laemmli buffer (Laemmli 1970) to extract total protein. Secreted proteins were precipitated as previously described (Vinatzer et al. 2005).

Yeast two-hybrid analysis.

The yeast strain AH109, containing the HIS3 and lacZ reporter genes, was used for yeast two-hybrid analyses (Matchmaker; Clontech, Palo Alto, CA, U.S.A.). The coding sequences of *avrPto*_{DC3000}, *avrPto*₈₃₈₋₁₆, and *avrPto*₈₃₈₋₁₆ (E95G) fragments were obtained by PCR amplification, cloned into the pGADT7 vector (activation domain), and sequence verified. The coding sequence of *Pto* from *S. pimpinellifolium* was cloned into the pGBKT7 vector and sequence verified. The positive control pGAL4 and the negative control pGBKT7 vector were all transformed into the yeast strain AH109 following the manufacturer's protocol. Protein expression was detected in transformed strains by immunoblotting. Transformants were dilution plated onto yeast potato dextrose agar and synthetic dextrose agar

lacking leucine/tryptophan/histidine containing 10 mM 3-aminotriazole. Transformants were also plated onto synthetic dextrose lacking leucine/tryptophan with α -galactosidase in the media for blue/white screening. Yeast growth was examined as previously described (Day et al. 2006).

Agrobacterium-mediated transient expression.

*avrPto*_{DC3000}, *avrPto*₈₃₈₋₁₆, and *avrPto*₈₃₈₋₁₆ (E95G) were cloned into pMD1 with a C-terminal fusion to the HA epitope using gateway technology. All clones were sequence verified and electroporated into *A. tumefaciens* C58C1.

A. tumefaciens C58C1 carrying a gene of interest expressed from the binary vector pMD1 was infiltrated onto *N. benthamiana* leaves at an OD₆₀₀ of 1.0 as described previously (Day et al. 2005). Leaf disks were harvested 40 h post inoculation for Western blot analyses. For transient expression in tomato, constructs were infiltrated onto the tomato genotypes Rio Grande 76R and Rio Grande *pto11* at an OD₆₀₀ of 0.1 (Salmeron et al. 1994). Transient expression assays were repeated at least three times with identical results.

Construction of the *P. syringae* 19 Δ *avrPtoB* null mutant.

The spectinomycin (Sp) resistance gene was used to replace the *avrPtoB* gene in *P. syringae* 19 by homologous recombination, creating *P. syringae* 19 Δ *avrptob*. A 1.5-kb promoter fragment upstream of the *avrPtoB* open reading frame (ORF) (UP) was PCR amplified and cloned into pENTR/D-TOPO creating pEDT (UP). A 1.2-kb fragment downstream of the *avrPtoB* ORF (DOWN) was PCR amplified and then cloned into pENTR/D-TOPO creating pEDT (DOWN). To create pEDT (Δ *avrPtoB*), the *EcoRI*/*AscI* fragment of pEDT (DOWN) was inserted into the same sites of pEDT (UP), creating pEDT (Δ *avrptob*). Then, a 1.5-kb Sp cassette was cloned into the *EcoRI* site between the upstream and downstream sequences in pEDT (Δ *avrptob*), creating pEDT (Δ *avrptob*:Sp). The Δ *avrptob*:Sp fragment was recombined into the suicide vector pLVC18-Rfc (gift from B. Staskawicz's lab) by a Gateway LR reaction (Invitrogen). pLVC18 (Δ *avrptob*:Sp) was moved into *P. syringae* 19 by electroporation. Strains were selected for growth on KB plates containing Sp at 50 μ g/ml and for loss of growth on KB plates containing tetracycline at 10 μ g/ml. PCR and sequence analysis confirmed that homologous recombination occurred at the *avrPtoB* locus.

P. syringae 19 Δ *avrptob*-1 was used to assess the virulence of *avrPto* alleles. *avrPto*₈₃₈₋₁₆, *avrPto*₈₃₈₋₁₆, *avrPto*_{DC3000}, and β -glucuronidase were cloned into the pBAV226 broad host range vector (Vinatzer et al. 2006) using gateway technology and electroporated into *P. syringae* 19 Δ *avrptob*-1. Growth curves were conducted as described above and repeated twice.

ACKNOWLEDGMENTS

This work was funded through University of California, Davis startup funds, United States Department of Agriculture National Research Initiative (award 2008-00712) and the hatch project CA-D*-PPA-7757-H to G. Coaker. S. Kunkeaw was funded by the Commission on Higher Education, Thailand. We thank G. Martin for providing antisera recognizing AvrPto and AvrPtoB, M. Davis for providing multiple *P. syringae* strains collected from 1996 to 2005, D. Mackey for providing *rps2-101c/rpm1-3 Arabidopsis* seed, R. Michelmore for providing tomato Rio Grande seed, and S. Y. He for providing the *P. syringae* DC3000 coronatine mutant.

LITERATURE CITED

Abramovitch, R. B., Kim, Y. J., Chen, S., Dickman, M. B., and Martin, G. B. 2003. *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO (Eur. Mol. Biol. Organ.) J. 22:60-69.

Abramovitch, R. B., Anderson, J. C., and Martin, G. B. 2006. Bacterial

elicitation and evasion of plant innate immunity. Nat. Rev. Mol. Cell Biol. 7:601-611.

Almeida, N. F., Yan, S., Lindeberg, M., Studholme, D. J., Schneider, D. J., Condon, B., Liu, H., Viana, C. J., Warren, A., Evans, C., Kemen, E., Maclean, D., Angot, A., Martin, G. B., Jones, J. D., Collmer, A., Setubal, J. C., and Vinatzer, B. A. 2009. A draft genome sequence of *Pseudomonas syringae* pv. *tomato* T1 reveals a type III effector repertoire significantly divergent from that of *Pseudomonas syringae* pv. *tomato* DC3000. Mol. Plant-Microbe Interact. 22:52-62.

Anderson, J. C., Pascuzzi, P. E., Xiao, F., Sessa, G., and Martin, G. B. 2006. Host-mediated phosphorylation of type III effector AvrPto promotes *Pseudomonas* virulence and avirulence in tomato. Plant Cell 18:502-514.

Arnold, D. L., Jackson, R. W., Waterfield, N. R., and Mansfield, J. W. 2007. Evolution of microbial virulence: The benefits of stress. Trends Genet. 23:293-300.

Arredondo, C. R., and Davis, R. M. 2000. First report of *Pseudomonas syringae* pv. *tomato* race 1 on tomato in California. Plant Dis. 84:371.

Boyes, D. C., Nam, J., and Dangel, J. L. 1998. The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc. Natl. Acad. Sci. U.S.A. 95 (26):15849-15854.

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. Cell 124:803-814.

Coplin, D. L., and Kado, C. I. 2001. A Laboratory Guide for the Identification of Plant Pathogenic Bacteria, 3rd ed., Vol. 1. N. W. Schaad, J. B. Jones, and W. Chun, eds. The American Phytopathological Society, St. Paul, MN, U.S.A.

Day, B., Dahlbeck, D., Huang, J., Chisholm, S. T., Li, D., and Staskawicz, B. J. 2005. Molecular basis for the RIN4 negative regulation of RPS2 disease resistance. Plant Cell 17:1292-1305.

Day, B., Dahlbeck, D., and Staskawicz, B. J. 2006. NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in *Arabidopsis*. Plant Cell 18:2782-2791.

Gassmann, W., Dahlbeck, D., Chesnokova, O., Minsavage, G. V., Jones, J. B., and Staskawicz, B. J. 2000. Molecular evolution of virulence in natural field strains of *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 182:7053-7059.

Göhre, V., Spallek, T., Häweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J. W., and Robatzek, S. 2008. Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr. Biol. 18:1824-1832.

Goode, M. J., and Sasser, M. 1980. Prevention of the key to controlling bacterial spot and bacterial speck of tomato. Plant Dis. 64:831-834.

Ham, J. H., Bauer, D. W., Fouts, D. E., and Collmer, A. 1998. A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. Proc. Natl. Acad. Sci. U.S.A. 95:10206-10211.

Harlow, E., and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Press., Cold Spring Harbor, NY, U.S.A.

Hauser, A. R., Cobb, E., Bodi, M., Mariscal, D., Valles, J., Engel, J. N., and Rello, J. 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit. Care Med. 30:521-528.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Janjusevic, R., Abramovitch, R. B., Martin, G. B., and Stebbins, C. E. 2006. A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. Science 311:222-226.

Jolley, K. A., Feil, E. J., Chan, M. S., and Maiden, M. C. J. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics 17:1230-1231.

Jones, J. D., and Dangel, J. L. 2006. The plant immune system. Nature 444:323-329.

Kim, Y. J., Lin, N. C., and Martin, G. B. 2002. Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. Cell 109:589-598.

King, E., Ward, M. K., and Raney, D. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lin, N. C., and Martin, G. B. 2005. An *avrPto/avrPtoB* mutant of *Pseudomonas syringae* pv. *tomato* DC3000 does not elicit Pto-mediated resistance and is less virulent on tomato. Mol. Plant-Microbe Interact. 18:43-51.

Lin, N. C., and Martin, G. B. 2007. Pto- and Prf-mediated recognition of AvrPto and AvrPtoB restricts the ability of diverse *Pseudomonas syrin-*

- gae* pathovars to infect tomato. *Mol. Plant-Microbe Interact.* 20:806-815.
- Lin, N. C., Abramovitch, R. B., Kim, Y. J., and Martin, G. B. 2006. Diverse AvrPtoB homologs from several *Pseudomonas syringae* pathovars elicit Pto-dependent resistance and have similar virulence activities. *Appl. Environ. Microbiol.* 72:702-712.
- Liu, J., Elmore, J. M., Fuglsang, A. T., Palmgren, M., Staskawicz, B., and Coaker, G. 2009. RIN4 functions with the plasma membrane H⁺-ATPase to regulate stomatal apertures during pathogen attack. *PLoS Biol.* 7:e1000139.
- Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Causant, D. A., Feavers, I. M., Achtman, M., and Spratt, B. G. 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. U.S.A.* 95:3140-3145.
- Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262:1432-1436.
- McCann, H. C., and Guttman, D. S. 2008. Evolution of the type III secretion system and its effectors in plant-microbe interactions. *New Phytol.* 177:33-47.
- Mindrinos, M., Katagiri, F., Yu, G. L., and Ausubel, F. M. 1994. The *A. thaliana* disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* 78:1089-1099.
- Mucyn, T. S., Clemente, A., Andriotis, V. M., Balmuth, A. L., Oldroyd, G. E., Staskawicz, B. J., and Rathjen, J. P. 2006. The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. *Plant Cell* 18:2792-2806.
- Mudgett, M. B., and Staskawicz, B. J. 1999. Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: Demonstration of secretion and processing during bacterial pathogenesis. *Mol. Microbiol.* 32:927-941.
- Nomura, K., Melotto, M., and He, S. Y. 2005. Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. *Curr. Opin. Plant Biol.* 8:361-368.
- Pedley, K. F., and Martin, G. B. 2003. Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. *Annu. Rev. Phytopathol.* 41:215-243.
- Pitblado, R. E., and Kerr, E. A. 1979. A source of resistance to bacterial speck—*Pseudomonas tomato*. *Tomato Genet. Coop. Rep.* 29:30.
- Pitblado, R. E., and Kerr, E. A. 1980. Resistance to bacterial speck (*Pseudomonas tomato*) in tomato. *Acta Hort.* 100:379-382.
- Rivas, L. A., Mansfield, J., Tsiamis, G., Jackson, R. W., and Murillo, J. 2005. Changes in race-specific virulence in *Pseudomonas syringae* pv. *phaseolicola* are associated with a chimeric transposable element and rare deletion events in a plasmid-borne pathogenicity island. *Appl. Environ. Microbiol.* 71:3778-3785.
- Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J., Sawa, T., Frank, D. W., and Wiener-Kronish, J. P. 2001. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J. Infect. Dis.* 183:1767-1774.
- Salmeron, J. M., Barker, S. J., Carland, F. M., Mehta, A. Y., and Staskawicz, B. J. 1994. Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition. *Plant Cell* 6:511-520.
- Salmeron, J. M., Oldroyd, G. E., Rommens, C. M., Scofield, S. R., Kim, H. S., Lavelle, D. T., Dahlbeck, D., and Staskawicz, B. J. 1996. Tomato Prf is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the Pto kinase gene cluster. *Cell* 86:123-133.
- Sarkar, S. F., and Guttman, D. S. 2004. Evolution of the core genome of *Pseudomonas syringae* a highly clonal, endemic plant pathogen. *Appl. Environ. Microbiol.* 70:1999-2012.
- Schulert, G. S., Feltman, H., Rabin, S. D., Martin, C. G., Battle, S. E., Rello, J., and Hauser, A. R. 2003. Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. *J. Infect. Dis.* 188:1695-1706.
- Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Micheltore, R. W., and Staskawicz, B. J. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* 274:2063-2065.
- Shan, L., He, P., Zhou, J. M., and Tang, X. 2000. A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *Mol. Plant-Microbe Interact.* 13:592-598.
- Shan, L., He, P., Li, J., Heese, A., Peck, S. C., Nurnberger, T., Martin, G. B., and Sheen, J. 2008. Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe* 4:17-27.
- Snedecor, G. W., and Cochran, W. G. 1980. Chapter 12 in: *Statistical Methods*, 7th ed. Iowa State University, Ames, IA, U.S.A.
- Sreedharan, A., Penalzo-Vazquez, A., Kunkel, B. N., and Bender, C. L. 2006. CorR regulates multiple components of virulence in *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant-Microbe Interact.* 19:768-779.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *24:1596-1599.*
- Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* 274:2060-2063.
- Vinatzer, B. A., Jelenska, J., and Greenberg, J. T. 2005. Bioinformatics correctly identifies many type III secretion substrates in the plant pathogen *Pseudomonas syringae* and the biocontrol isolate *P. fluorescens* SBW25. *Mol. Plant-Microbe Interact.* 18:877-888.
- Vinatzer, B. A., Teitzel, G. M., Lee, M. W., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J., and Greenberg, J. T. 2006. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol. Microbiol.* 62:26-44.
- Wichmann, G., Ritchie, D., Kousik, C. S., and Bergelson, J. 2005. Reduced genetic variation occurs among genes of the highly clonal plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*, including the effector gene *avrBs2*. *Appl. Environ. Microbiol.* 71:2418-2432.
- Wulf, J., Pascuzzi, P. E., Fahmy, A., Martin, G. B., and Nicholson, L. K. 2004. The solution structure of type III effector protein AvrPto reveals conformational and dynamic features important for plant pathogenesis. *Structure* 12:1257-1268.
- Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J., and Zhou, J. M. 2008. *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr. Biol.* 18:74-80.
- Xing, W., Zou, Y., Liu, Q., Liu, J., Luo, X., Huang, Q., Chen, S., Zhu, L., Bi, R., Hao, Q., Wu, J. W., Zhou, J. M., and Chai, J. 2007. The structural basis for activation of plant immunity by bacterial effector protein AvrPto. *Nature* 449:243-247.
- Yan, S., Liu, H., Mohr, T. J., Jenrette, J., Chiodini, R., Zaccardelli, M., Setubal, J. C., and Vinatzer, B. A. 2008. Role of recombination in the evolution of the model plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000, a very atypical tomato strain. *Appl. Environ. Microbiol.* 74:3171-3181.
- Yang, B., Sugio, A., and White, F. F. 2005. Avoidance of host recognition by alterations in the repetitive and C-terminal regions of AvrXa7, a type III effector of *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant-Microbe Interact.* 18:142-149.
- Yeom, I., Nguyen, H. P., and Martin, G. B. 2009. Phosphorylation of the *Pseudomonas syringae* effector AvrPto is required for FLS2/BAK1-independent virulence activity and recognition by tobacco. *Plant J.* 61:16-74.
- Zhou, J. M., and Chai, J. 2008. Plant pathogenic bacterial type III effectors subdue host responses. *Curr. Opin. Microbiol.* 11:179-185.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Plant-Associated and Environmental Microbes database: www.pamdb.org